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A METHOD OF TITRATING PROTEOLYTIC ENZYMES

BY FREDERICK L. GATES

(From the Laboratories of The Rockefeller Institute for Medical Research)

In 1927[1] the author published a brief note on a method of enzyme titration, which was later somewhat modified and demonstrated at the 1928 meeting of the Federation of American Societies for Experimental Biology. Since it has not yet been found possible to elaborate the technique, with protocols, in a longer article, as had been hoped, it seems best to publish these suggestions now and so to make them generally available for adaptation to individual problems in enzyme research.

The method is based on a reduction in the density of pieces of exposed photographic film by the release of silver through the progressive proteolysis of the gelatin layer. The relative densities before and after exposure to the enzyme solution are read against a suspension of the same gelatin-silver emulsion in a Duboscque type colorimeter, or by means of a photometer.

The film: Eastman Commercial, 8″ x 10″; 2 to 6 in a pile, irradiated by Roentgen ray, 50,000 v., 10 m.a., 25″ target distance, 2 min. exposure. (Small rectangles may be defined and numbered by lead strips and figures cemented to the cassette.) The exposed film is fully developed, fixed in plain hypo (no hardener), washed, dried, rewashed, dried again and cut into rectangles 2 x 2.5 cm.

The cells: No. 14 (1.5 mm) copper wire coiled around a 1.3 cm rod is snipped off in nearly complete circles (0.5 cm opening), bent flat and sealed with paraffin on 1.5 x 2 cm glass slips, with the opening on a 2 cm side. Backs are glass slips 1.5 x 2 cm. Cell, film and back are clipped together with a spring clothes-pin.

The colorimeter: A Klett, Bausch and Lomb or other Duboscque type is used, with spring clips under the tube shelves to hold the film carriers. These carriers are double leaves of thin brass, lacquered flat black, with centered 1 cm holes, between which the film is slipped for insertion under the shelf in the light path. The suspension for comparison is made of gelatin-silver emulsion, dissolved off of two films in hot water. Glycerin is added to 50 per cent. to delay sedimentation. A completely cleared film is used in the carrier under this tube. Fifty per cent. glycerin solution fills the tube above the test films. Both tubes must be at the same level when readings are made.

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[1] *Proc. Soc. Exp. Biol. and Med.*, 24: 936, 1927.

Method of use: Readings are made on each film before use, one film being reserved as a control. The others are each placed between a glass back and the copper ring of a cell, gelatin side to copper ring, and the whole held together with a spring clothes-pin. Enzyme solution is filled into the cell with a capillary pipette, and the cell placed upside down, gelatin film surface forming the roof, at constant temperature for a carefully timed interval. (A separate film is used for each determination desired.) Then the clothes-pin is released, the film is rinsed quickly in cold water and dried rapidly, clipped by one corner, before a fan. Units should be started at not less than fifteen-second intervals to allow for this rinsing of the successive films. When dry the density of each film is again read against the gelatin-silver suspension. Any change in the density of this suspension is revealed by a recheck on the control film, and the other control readings are corrected accordingly. Results are obained in percentage of gelatin unaffected—the ratio of the final to the (corrected) control reading on each film. 100 minus this ratio, (*i.e.*, the percentage of gelatin affected) is proportional to the enzyme activity at the time and under the conditions of the test.

[Reprinted from THE JOURNAL OF GENERAL PHYSIOLOGY, September 20, 1930,
Vol. 14, No. 1, pp. 31–42]

A STUDY OF THE BACTERICIDAL ACTION OF ULTRA VIOLET LIGHT

III. THE ABSORPTION OF ULTRA VIOLET LIGHT BY BACTERIA

BY FREDERICK L. GATES

(From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Department of General Physiology, Harvard University, Cambridge)

(Accepted for publication, June 10, 1930)

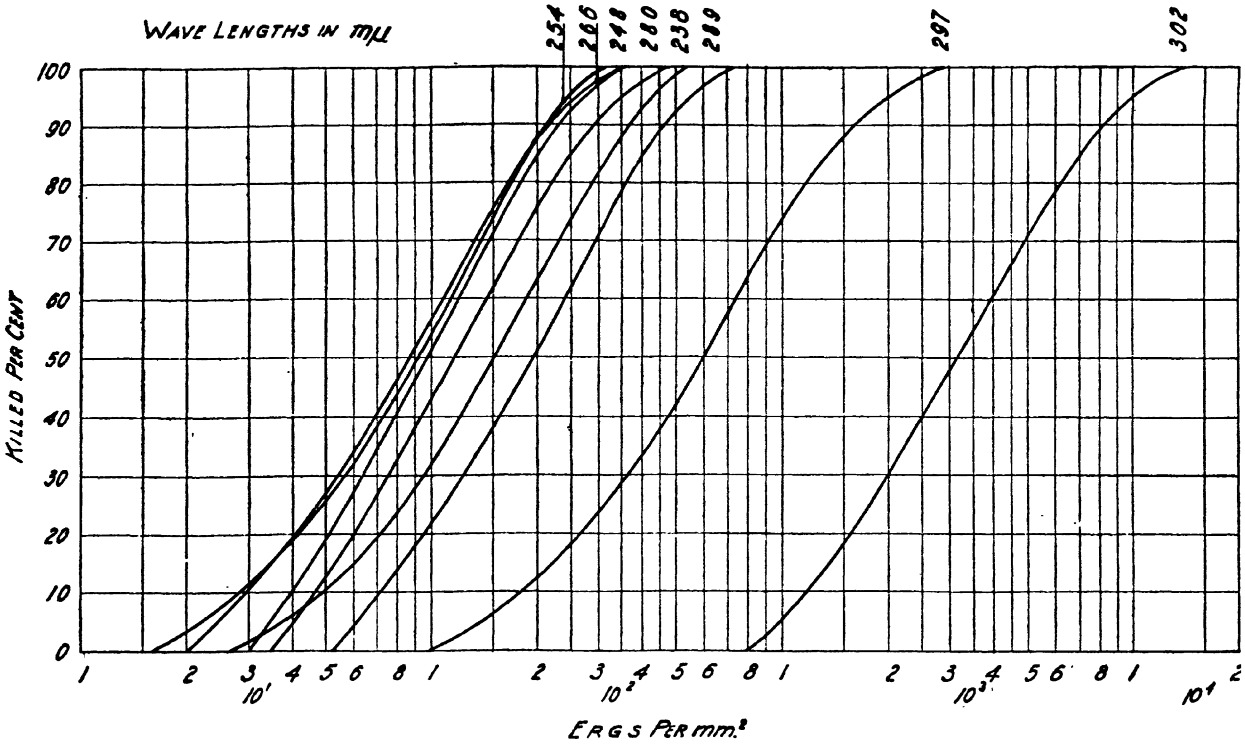
In this study of the bactericidal action of ultra violet light the first paper (1) described the reaction of an 18 hour culture of *Staphylococcus aureus* to monochromatic radiations. It was shown that the course of the reaction among large numbers of organisms was approximately the same at each wave length studied but that widely different incident energies were required at different wave lengths to produce these similar effects.

The second paper (2) discussed the limits of the bactericidal zone, showed that the reaction had a low temperature coefficient, (approximately 1.1), gave evidence that within the variations of the methods used no significant errors were introduced by differences in the measured intensity of the source or in the hydrogen ion concentration of the medium, and indicated that plane polarization of the incident light had no effect upon the reaction.

The present paper deals with the absorption of ultra violet light by intact bacteria. A final paper in this series will discuss structural and chemical units of bacterial protoplasm that may prove to be involved in the reaction which results in the organism's death.

Incident Energy Relationships at Various Wave Lengths

Text-fig. 1, reproduced from the first paper of this series, shows that although the course of the bactericidal reaction was approximately the same at each wave length studied, these similar curves were found at very different incident energy levels at different points in the ultra violet spectrum.



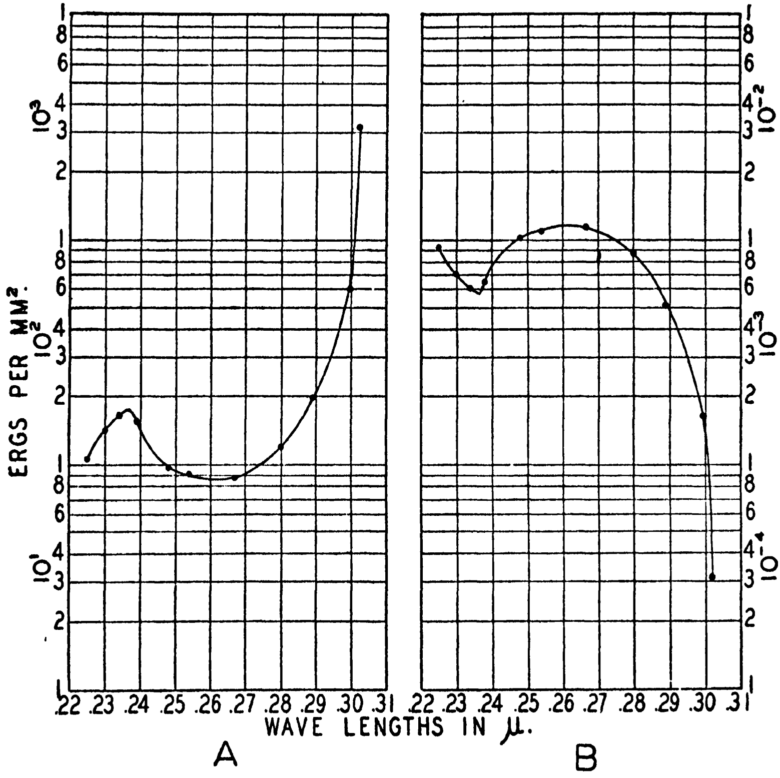
Text-Fig. 1. Incident energies required for bactericidal action at various wave lengths in the ultra violet spectrum

For example, if the incident energy required to kill half the exposed staphylococci be taken as an index, this energy requirement ranged from 3,150 ergs per mm.^{2} at 302 m.μ, a wave length near the limit of bactericidal action, to 88 ergs at 266 m.μ. At other wave lengths, either above or below 266 m.μ, more incident energy was required. The destruction of 50 per cent of the microorganisms is chosen as the index because it is in the most accurately determined part of the curves where the mortality rate is least affected by variations in individual resistance.

If the incident energies involved in 50 per cent destruction be plotted and joined by a continuous line the resulting curve appears as in Text-fig. 2A. Parallel experiments on an 18 hour culture of *Bacillus coli* gave bactericidal energy curves similar in trend to those for *S. aureus*, and although complete statistics were not obtained on this bacterium the middle or exponential portion of the lethal reaction curves was determined by repeated observations at each wave length studied. Text-fig. 3A shows the incident energies involved in the destruction of 50 per cent of the exposed *coli* organisms. Its essential similarity to the corresponding *aureus* curve is apparent. Both curves would probably be somewhat modified in detail if more wave lengths were available for study.

These characteristic curves (Text-figs. 2A and 3A) show clearly that less incident energy is required between 260 and 270 m.μ than in any other region of the bactericidal zone examined, and point toward a second minimum below 230 m.μ. The presence of a sharp peak in the energy requirement near 240 m.μ appears to be equally significant. Due apparently to the use of but a few wave lengths in the bactericidal zone, or to failure to measure spectral intensities, or to crude methods of estimating bacterial destruction, the occurrence of a minimum at about 266 m.μ, and of the peak in the curve near 240 m.μ has been overlooked by most investigators. Usually they have been content with the conclusion that the shorter the wave length the more marked the bactericidal action (3).

Bang (4) was apparently the first to observe regions of special bactericidal "effectiveness" or of corresponding bacterial susceptibility. Using a 30 ampere carbon arc through a spectrograph at 20 m.μ intervals, and estimating bactericidal efficiency mainly by relative exposure, he found two regions of maximal action, an "inner maximum" between 340 and 360 m.μ, and an "outer maximum" between 240 and 260 m.μ. The lethal exposure varied from 1920 seconds at 330 to 300 m.μ through 120 seconds at 300 to 280 m.μ to 4 seconds at 280 to 260 m.μ and 2 seconds at 260 to 240 m.μ. Then longer exposures were required. The zone from 240 to 220 m.μ needed 20 seconds, that between 220 and 210 m.μ, 30 seconds, and that between 210 and 200 m.μ required 120 seconds exposure to kill the organisms.



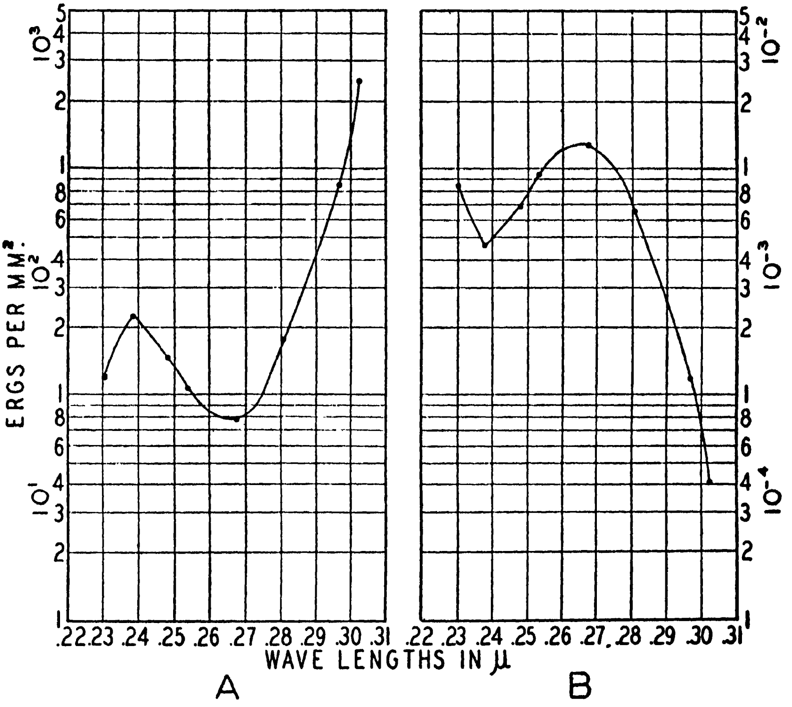
TEXT-FIG. 2. A. Curve of incident energies involved in the destruction of 50 per cent of *S. aureus*.

B. Curve of the reciprocals of 2A.

These longer exposures at short wave lengths were evidently due to the rapid decrease in intensity of the source employed. Mme. Henri (3) thought that both of Bang's maxima should be ascribed to variations in intensity of his carbon arc.

Newcomer (5) exposed *B. typhosus* in quartz capillary tubes to narrow bands of the iron arc spectrum and counted surviving bacteria plated in agar, after exposures of 5 or 10 minutes. His figures, like Bang's, indicate a region of maximum effect between 254 and 268 m.μ, but his iron arc varied so much in spectral intensity that he did not find any significance in this peak of effectiveness. He concluded that "equal intensities produce equal effects in the regions 2100–2800. If there is a maximum in this region it is at most only slight and would be in the neighborhood of 2600."

When Mashimo (6) varied the exposure of bacteria in a spectrograph from 15 seconds to 80 minutes he found the first evidence of bactericidal action at 275 m.μ.



Text-Fig. 3. A. Curve of incident energies involved in the destruction of 50 per cent of *B. coli*.

B. Curve of the reciprocals of 3A.

With somewhat longer exposures the zone widened rapidly so that with a 3 minute exposure it extended from below 210 to above 280 m.μ. The marked action at 275 m.μ was evidently due to a relatively high intensity of his source at this wave length, as an examination of his published photograph shows.

Thus variations in spectral intensity, with no adequate methods of measurement or control, made it difficult to interpret the maxima found by Bang, Newcomer, and Mashimo, and these authors laid no stress upon them.

In the present study the measurement of the monochromatic radiant energy in absolute units focuses attention upon the marked differences in the incident energies required at different wave lengths to kill *S. aureus* and *B. coli.* The shape of the energy curves for 50 per cent destruction immediately suggests that it is the specific absorption of the ultra violet radiations that gives the curves significance. This relation of incident energy to its specific absorption is made the more striking by plotting the reciprocals of these energies (Text-figs. 2B and 3B), by which graphs not unlike absorption curves, with maxima at 266 and beyond 230 m.μ are produced.

As a first step in the further analysis of the bactericidal reaction it is obviously necessary to compare these curves with those for the absorption of ultra violet energy by the corresponding bacteria.

The Absorption of Ultra Violet Light by Bacteria

Apparently studies of the absorption spectra of bacteria have been confined hitherto to bacteria in suspension in a fluid medium (7, 8). Suspensions in liquids are unsuitable for such examinations. Reflection and refraction from the bacterial bodies, with consequent scattering of light so that bacterial suspensions are opaque even in the visible region of the spectrum, and the difficulty of estimating the number of organisms traversed, frustrate any attempt to obtain results of quantitative significance.

But a loopful of bacteria may be taken *en masse* from the surface of an agar slant and pressed between quartz plates into a layer so thin that is is all but colorless in visible light, and so transparent that objects may be seen through it clearly and without distortion. The bacteria are in optical contact and form a homogeneous medium for the transmission of visible or ultra violet light. Such a film is composed almost entirely of bacterial cells and the immediate products of their metabolism. Tests show that films of like thickness of the agar medium from which the bacteria were removed absorb no significant amounts of ultra violet light.

Such a film of bacteria may be set up in one optical path of a quartz photometer, with similar plates of quartz and a drop of glycerol in the other path as a control, and the absorption coefficient of the organisms readily obtained in the manner commonly employed for chemical solutions (9).

Two beams of light from the same source, which have passed through the specimen under examination or its control in the photometer, are spread by a quartz spectrograph into parallel spectra in the plane of a photographic plate. The energy that traverses the control path is subject to quantitative variation by means of a sector shutter, and with the shutter set at predetermined openings a series of photographs is made. Then the point, or points, of equal blackening in each pair of spectral photographs indicate the wave lengths at which the test specimen and the sector shutter in the control path have reduced the original intensity of the light to the same degree.*

In such experiments it is necessary to know accurately the depth of the medium traversed in order to calculate the coefficients of absorption for a layer of unit thickness. The standard or unit of thickness in these observations was chosen as 0.8 μ, the average diameter of *S. aureus* (10), so that the coefficients of absorption were obtained for a single layer of bacteria. The shape of *B. coli* and its wide variations in size precluded even so crude an estimate of a "single layer," so the coefficients for *B. coli* were figured arbitrarily for a layer of the same thickness (0.8 μ) to permit a comparison with the results for *S. aureus*.

The thickness of these films of bacteria between quartz plates was found to lie between 5 and 15 μ. Since the method of measurement employed (11) is easy, and is accurate (in microns) to the second or third decimal place it may be described in brief. The method is based on interferometry, namely, the measurement of the interference bands formed by the coincident spectra of white light reflected into a wavelength spectroscope from the two quartz surfaces enclosing the bacterial film.

A point source of white light (carbon arc, concentrated tungsten filament, or even a flashlight bulb with the filament vertical), is set up at a measured angle of incidence (60°) to the film specimen, so that its light is reflected from the back surface of the first quartz plate and from the front surface of the second (the surfaces enclosing the film), into a wavelength spectroscope. The reflecting surfaces must be chosen near the bacterial film, but not to include it, since adequate reflection occurs only when air is the medium between the two plates. With a proper set-up, and films of suitable thickness a series of vertical interference bands will cut across the spectrum.

---

* Measurements of absorption by such films made in 1923 with thermocouple and galvanometer (*Proc. Soc. Exp. Biol. and Med.*, 1923, 21, 61), were evidently not as accurate as those obtained by the present method and have been discarded.

Then the distance (t) between the two plates (the thickness of the included film) is found by noting the wave lengths (λ_1 and λ_2) between which any convenient number (n) of interference bands is counted for substitution in the following equation:

$$t = \frac{n\,\lambda_1\,\lambda_2}{2\,\mu\,\cos r\,(\lambda_1 - \lambda_2)}$$

μ is the refractive index of the medium, and r is the angle of incidence and reflection. Since air is the medium $\mu = 1$, and with r at 60° $\cos r = 0.5$ so that the equation becomes

$$t = \frac{n\,\lambda_1\,\lambda_2}{(\lambda_1 - \lambda_2)}$$

For example, with the apparatus set up as described 5 interference bands are counted between $\lambda_1 = 7241$ Å u. and $\lambda_2 = 4638$ Å u. It is only necessary to multiply these waves lengths together, divide by their difference, and multiply the result by 5 to determine that the distance between the plates *in the area examined* is 6.45 μ or 0.00645 mm.

In these experiments the films of bacteria, pressed out by hand, were not strictly plane parallel, but often thicker at one edge than at the opposite one, so four readings were taken at 90° intervals around the rim of each film and combined for an average thickness. The differences in thickness in the small central area exposed in the photometer were not so great as to make it desirable to obtain a mean exponentially according to Lambert's law. For example:

Film 1. *B. coli.* 90° readings at edge of film: 5.58, 6.17, 5.63, and 5.66 μ. Average 5.76 μ.

Film 2. *S. aureus.* 90° readings at edge of film: 9.21, 11.66, 11.24, and 10.53 μ. Average 10.66 μ.

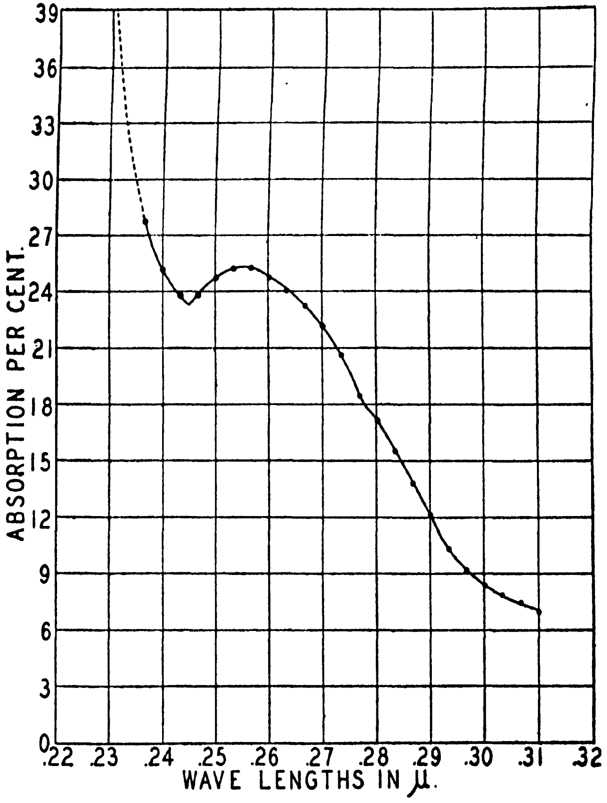
Given the average thickness of each film, the number of layers (n) of bacteria it contained was then available for the determination of the coefficients of absorption for a layer 0.8 μ thick. For this purpose the familiar equation of Lambert's law was used, to which Wood (11) says that no exception has ever been found that was not attributable to experimental error. Thus if a is the fraction of the original intensity (I_o) transmitted by each unit layer, the intensity (I_t) observed after passage through n layers is given by the equation:

$$I_t = I_o\,a^n$$

The relation of I_t to I_o is chosen at appropriate intervals on the sector shutter of the quartz photometer, as described above, and this equation solved for a gives the coefficient of transmission at the wave length where the two spectra match. Then $1-a$ is the corresponding coefficient of absorption for a single layer of bacteria 0.8 μ thick.

The mean absorption curve from five series of determinations with *S. aureus* is given in Text-fig. 4, and from five series with *B. coli* in Text-fig. 5. The curves are characteristic, and similar to those found for various biological tissues and fluids containing proteins or protein derivatives (12, 13, 14) on which their main features evidently depend. Differences in the curves for the two organisms are apparent, but in view of the experimental errors involved in such determinations they probably should not be stressed.

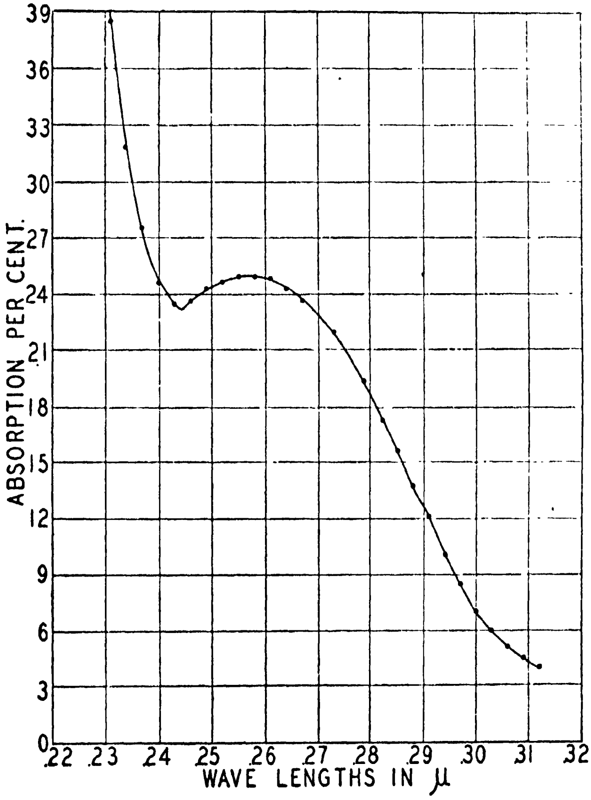
The general similarity of these absorption curves to the reciprocals of the curves for bactericidal incident energy is obvious. All four curves rise rapidly from low levels beyond 300 m.μ to a maximum between 260 and 270 m.μ, then drop to a minimum near 240 m.μ and rise again toward a limit beyond the range of experimental observation. One is tempted to correct the incident energies involved in the bactericidal reaction by these absorption coefficients for the entire organisms in order to obtain an approximation of the total energies absorbed. Yet the two sets of curves show important points of difference also, especially in the location of the dip near 240 m.μ, and a closer consideration of their relationship indicates that such a quantitative correction would be futile. The sum of the absorption coefficients of all the chemical entities in the bacterial cell cannot be expected exactly to correct the wide differences found in the bactericidal incident energies at different wave lengths unless every chemical group is involved in the bactericidal reaction in exact proportion to its contribution to total absorption. Such a hypothesis is hardly tenable. It seems more probable that it is the effect of ultra violet energy on a single vital and sensitive structural or chemical unit that results in subsequent failure in cell multiplication. Rahn (15) has recently figured from the curves of abiotic reactions among large numbers of single cell organisms that death of the cell probably involves only a single chemical entity. As Coblentz and Fulton (16) have suggested, it is to be presumed that only a small fraction of the total absorbed energy first affects such an essential structure, and so leads to the death of the cell. Neither the reciprocals of the energy curves, which are undoubtedly modified by the absorption of light by elements not involved in the



TEXT-FIG. 4. The coefficients of absorption of ultra violet light by a layer of *S. aureus* 0.8 μ thick.

bactericidal reaction, nor the curves for total absorption give an accurate picture of the absorption curve of this vital substance, whatever it may be. Yet their similarities and their differences are alike useful in the further search for such an essential and sensitive element in the cell's structure and economy.

Thus it may be predicted that there are a number of chemical entities or aggregates in the living cell which have rather similar coefficients of ultra violet absorption, and that the sum of these similar absorption curves largely determines the shape of the curve for the entire cell.



Text-Fig. 5. The coefficients of absorption of ultra violet light by a layer of *B. coli* 0.8 μ thick.

Among these substances will be found the one essential element first affected by ultra violet light in the bactericidal reaction, and its absorption curve will be similar to, though not identical with, the reciprocal of the lethal energy curve. Finally it seems improbable that this sensitive substance is uniformly distributed throughout the cell's protoplasm. An examination of the evidence for its concentration in the cell nucleus, and the further search for evidence of its chemical character are reserved for the final paper of this series.

SUMMARY

The simple conclusion of former investigators that the shorter the wave length of ultra violet light the greater the bactericidal action is in error. A study with measured monochromatic energy reveals a characteristic curve of bactericidal effectiveness with a striking maximum between 260 and 270 m.μ. The reciprocal of this abiotic energy curve suggests its close relation to specific light absorption by some single essential substance in the cell.

Methods are described for determining the absorption curve, or absorption coefficients, of intact bacteria. These curves for *S. aureus* and *B. coli* have important points of similarity and of difference with the reciprocals of the curves of bactericidal incident energy, and point the way in a further search for the specific substance, or substances, involved in the lethal reaction.

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EXPERIMENTS ON ANAPHYLAXIS TO AZOPROTEINS
THIRD PAPER

BY K. LANDSTEINER, M.D., AND PHILIP LEVINE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, May 23, 1930)

In preceding papers (1, 2) it has been shown to be possible to induce anaphylaxis in guinea pigs by injecting azoproteins, namely, horse serum coupled with diazotized *p*-arsanilic acid. Animals treated in this way become sensitive to azoproteins containing the same azocomponent but another sort of protein; thus the reactions obviously depend upon the arsanilic acid group.

These results have been confirmed by Meyer and Alexander (3) who used also the method of passive sensitization, and by Klopstock and Selter (4). Some of the observations of these workers will be discussed later. Another investigation on the subject has been made recently by Tillett, Avery and Goebel (5). These authors employed as antigens azoproteins prepared by combining globulin or albumin with diazotized glucosides.

The present study was undertaken with the purpose of investigating the specificity of the anaphylactic reactions and of gaining further information on the inhibition phenomenon produced by simple chemical substances (2). In order to test the specificity of anaphylaxis to azoproteins, antigens were selected which differed only in the steric configuration of the specifically reacting groups. The fact that antigens containing sterically isomeric groups are serologically different has already been shown by means of the precipitin reaction by Landsteiner and van der Scheer (6, 7) for *l*- and *d*-phenyl (*p*-aminobenzoylamino) acetic acids and *l*-, *d*-, and *m*-tartaric acids. Similar results were obtained by Avery and Goebel (8, 9) who used as antigens glucosides containing glucose or galactose.

EXPERIMENTAL

Specificity of the Anaphylactic Reaction.—For the following experiments antigens were used containing the radicals of the *d*- and *l*-tartaric acids. These were prepared by coupling horse serum with *d*- and *l*-*p*-aminotartranilic acid in the manner previously described (7).

For the sensitization guinea pigs were injected intraperitoneally three times at weekly intervals with a suspension of 1 cc. of the antigens containing about 5 per cent protein. The animals weighed 210 to 250 gm.; the injections were well tolerated.

The solutions used for the reinjection were prepared in the same manner as the sensitizing antigens except that chicken serum was employed instead of horse serum and that after precipitation with acid, the azoproteins, without treatment with alcohol, were brought in solution with the aid of sodium carbonate; the solutions were made isotonic and adjusted to litmus neutrality. The stock solutions of the antigen were brought to a protein content of 3.5 per cent. The test injections were made intravenously 3 weeks after the last administration of the sensitizing dose, with 1 cc. of various dilutions or a larger volume of the concentrated antigen. At the time of the test the weight of the animals was about 400 gm. The results of an experiment in which the specificity of the reaction was tested are given in Table I.

From Table I it is seen that, with one possible exception, the sensitization succeeded regularly; one animal showed only slight symptoms. The quantity sufficient for inducing shock was as low as 0.35 to 0.7 mg. The symptoms were in all cases typical of anaphylactic shock, and in the animals which died, the lungs were distended and the heart was beating. The reactions were strikingly specific since an injection of about 50 to 100 minimal lethal doses was innocuous for the animals sensitized to the heterologous antigen, apart from a drop in temperature which generally did not exceed 1°. In this respect the results are in full agreement with those reported by Tillett, Avery, and Goebel (5).

A further proof of the specificity of the reactions was furnished by reinjecting, on the following day, some of the animals which had received a dose of the heterologous antigen without showing symptoms of anaphylactic shock. Such animals, with one exception, reacted to a subsequent injection of the same quantity of the homologous antigen, although there was evidence of some protection (Table II).

TABLE I[1]

Animals Sensitized with d-Antigen

Tested with *d*-antigen				Tested with *l*-antigen			
Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms
		°*C.*				°*C.*	
1	70		† 4 min.	14	70	−0.7	Negative
2	35		† 3 "	15	70	−0.4	"
3	35	−4.3	Severe	16	35	−1.5	Slight
4	35		† 3 min.	17	35	−0.7	Negative
5	35		† 3 "	18	17.5	−0.4	"
6	35		† 3 "	19	8.8	−0.9	"
7	35		† 5 "	20	0.7	+0.9	"
8	17.5	−3.3	Severe				
9	17.5		† 4 min.				
10	8.8		† 3 "				
11	1.5		† 4 "				
12	0.7	−2.3	Very severe				
13	0.35	−0.6	Moderate				

Animals Sensitized with l-Antigen

Tested with *d*-antigen				Tested with *l*-antigen			
Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Quantity of azo-protein injected in mg.	Subsequent change in body temperature	Result, symptoms
		°*C.*				°*C.*	
21	70	+1.0	Negative	26	70		† 4 min.
22	35	−1.1	"	27	35	−2.8	Slight
23	8.8	−1.9	"	28	35		† 3 min.
24	3.0	−0.5	"	29	8.8		† 3 „
25	0.7	−0.6	"	30	3.0		† 5 "
				31	0.7		† 5 "
				32	0.35	−2.4	Severe
				33	0.18	−1.3	Slight

[1] The designations correspond to those in the previous paper (7).

† Death of animal.

Guinea pigs which were sensitized with only one injection of 1 cc. of the antigen showed the same degree of sensitivity as those in the experiment reported in Table I since they also succumbed to a shocking dose of 0.7 mg. An example is given in Table III.

TABLE II

Reinjection Experiments

Animals Sensitized with d-Antigen

Guinea pig No.	Quantity of *l*-antigen injected in mg.	Result, symptoms	Quantity of *d*-antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
				°*C.*	
17	35	Negative	35	−1.8	Negative
18	17.5	"	17.5		† 5 min.
19	8.8	"	8.8	−2.5	Severe
20	0.7	"	0.7		† 8 min.

Animals Sensitized with l-Antigen

Guinea pig No.	Quantity of *d*-antigen injected in mg.	Result, symptoms	Quantity of *l*-antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
				°*C.*	
21	70	Negative	70		† 8 min.
22	35	"	35		† over night
24	3	"	3	−2.2	Severe
25	0.7	"	0.7		† 5 min.

TABLE III

Guinea pigs weighing 200 to 220 gm. were given one intraperitoneal injection of *d*-antigen: reinjection with shocking antigen at the end of 23 days when the animal weighed about 300 gm.

Guinea pig No.	Quantity of *d*-antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
		°*C.*	
34	3		† 3 min.
35	0.7		† 4 "
36	0.7		† 5 "
37	0.35	−0.7	Moderate to severe

The above experiments furnished hardly any evidence of the inhibition of the anaphylactic reaction by the administration of large doses of the shocking antigen, which was observed by Klopstock and Selter (4). However there was some indication of the zone phenomenon in experiments (Table IV) in which animals were sensitized with an antigen made by coupling beef serum with diazotized arsanilic acid as was done by Klopstock and Selter.

It is seen from Table IV that only one of three animals was killed by the largest dose employed while four out of five succumbed to a dose four times smaller.

TABLE IV

Eleven guinea pigs were sensitized by one subcutaneous injection of atoxyl beef antigen (1 cc. = 16.6 mg.) which was purified by means of acid and alcohol; the reinjection with atoxyl chicken antigen prepared in the same manner as the beef antigen was made 16 days after the sensitization. The reinjection was made intravenously in a volume of 1 cc.

Guinea pig No.	Quantity of chicken antigen injected in mg.	Subsequent change in body temperature	Result, symptoms
		°C.	
38	6	−6.2	Very sick
39	6	−1.1	Negative
40	6		† 5 min.
41	1.5	−1.8	Slight to moderate
42	1.5		† 4 min.
43	1.5		† 7 "
44	1.5		† 7 "
45	1.5		† 6 "
46	0.5	−1.3	Slight to moderate
47	0.5		† 5 min.
48	0.5		† 6 "

Sensitization with Antigens Prepared According to the Method of Klopstock and Selter.—Whilst in the first experiments on anaphylaxis to azoproteins, the antigens used for sensitization were isolated after coupling in alkaline solution by precipitation with acid, Klopstock and Selter sensitized guinea pigs by injecting guinea pig serum to which they added neutralized diazosolutions. The reinjections were made with azoproteins prepared from guinea pig serum according to the older method. In the experiment to be described (Table V) the procedure of Klopstock and Selter was followed.

The results as judged from the reinjection with the chicken serum preparation confirmed in a general way those of Klopstock and Selter except that the sensitization did not succeed regularly. They differed in that most of the animals tested did not react to the guinea pig serum preparation. No attempt was made to inquire into the cause of this discrepancy.

TABLE V

Eighteen guinea pigs were sensitized by a subcutaneous injection of 1 cc. of a solution made by adding two volumes of 1 per cent neutralized solution of diazotized *p*-arsanilic acid to one volume of fresh guinea pig serum (4). The solution stood overnight in the ice box before injections were made. The reinjections were made after an interval of 33 days.

Reinjection with chicken *p*-arsanilic acid antigen (1 cc. = 18.6 mg. protein)				Reinjection with guinea pig *p*-arsanilic acid antigen (1 cc. = 23.8 mg. protein)			
Guinea pig No.	Quantity of antigen in mg.	Subsequent change in body temperature	Result, symptoms	Guinea pig No.	Quantity of antigen in mg.	Subsequent change in body temperature	Result, symptoms
		°C.				°C.	
49	9.0		† 4 min.	59	12	−1.2	Negative
50	9.0	−1.3	Slight	60	12	−0.4	"
51	5.0		† 3 min.	61	5	−1.5	Slight to moderate
52	5.0		† 20 "	62	5		† 4 min.
53	5.0	−0.3	Negative	63	5	−0.2	Negative
54	5.0		† 20 min.				
55	5.0	−1.0	Negative				
56	1.5	−0.3	"	64	1.5	−0.6	"
57	1.5		† 6 min.	65	1.5	−1.8	"
58	1.5	−0.8	Negative	66	1.5	−0.6	"

From their observations Klopstock and Selter conclude that for the sensitization and the production of antibodies, as well as for the reactions *in vitro*, it is not necessary to have a chemical combination of the azocomponents with protein but that it suffices to use simple "mixtures" of diazocompounds and protein. They stress the view that the diazocompounds would, by themselves, act as antigens and the proteins only enhance the antigenic activity which is inherent in the simple substances (4, 10, 11). However, as has been shown by Heidelberger and Kendall (12) and one of the present writers (13), there undoubtedly takes place a combination of the diazocompounds with proteins also in neutral solution under the conditions of the method of Klopstock and Selter. Consequently their procedure does not involve a new principle but must be looked upon as a modification of the older method of preparing azoproteins. It is true that in a footnote to their last publication[1] the authors make a statement which may be interpreted as an admission that they dealt not with mixtures but with chemical combinations. As for those instances in which Klopstock and Selter succeeded in sensitizing with diazocompounds alone, it can be assumed that these substances combined with the proteins of the animal injected, so that in this case also the immunization is probably attributable to an azoprotein. Indeed it has been shown that animals can be immunized by azoproteins the protein part of which is derived from their own species (14).

The phenomenon observed by Klopstock and Selter, that guinea pigs sensitized with diazosolutions alone exhibit skin reactions on intradermal injections of the same diazocompound, may be due to a special mechanism if further study should show that the skin reactions can be induced in this way only and not by sensitization with azoproteins. One may suppose either that the chemical combinations formed in the body on the injection of diazosolutions are different from those prepared *in vitro* or that the sensitization is brought about by the direct action on tissues (skin) by the diazocompound as such. Even in the latter case, because of the ease with which diazosolutions combine with proteins it would not be justifiable to draw conclusions, from the experiments discussed, upon the possibility of sensitization with simple chemical substances in general, particularly those which do not readily form compounds with proteins.

Inhibition of Anaphylactic Shock by Simple Substances.—In the experiments of Landsteiner (2) a peculiar phenomenon was noticed, *i.e.* shock could be prevented by injecting sensitized animals with simple azocompounds containing the same specific group as the sensitizing antigen. The substances used were compounds prepared by coupling diazotized arsanilic acid with tyrosine or *p*-oxybenzoic acid. Analogous results were described by Klopstock and Selter (4) with the sodium salts of *p*-arsanilic acid (atoxyl) and *m*-aminobenzensulfonic acid. K. Meyer did not succeed in obtaining antianaphylaxis by injecting atoxyl into animals sensitized to azoproteins prepared from *p*-arsanilic acid.

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[1] See (4), page 465.

Similar experiments were carried out by Tillett, Avery, and Goebel (5) with guinea pigs sensitized to azoproteins containing glucosides. When these animals were injected with uncombined glucoside immediately prior to the administration of the antigen, shock could be prevented. If, however, the shocking injection was given 2 hours later protection was no longer demonstrable. Consequently the authors raise the question as to the mechanism of this inhibitory effect which, indeed, can hardly be looked upon as a desensitization on account of its transitory nature.

Our present experiments were carried out with a series of animals sensitized with antigens prepared from *d*- and *l*-*p*-aminotartranilic acid and another series sensitized to azoproteins prepared from *p*-arsanilic acid.

Guinea pigs weighing 200 to 250 gm. were sensitized as in the previous experiment (see Table I) and were tested 3 weeks after the last injection. At various intervals before the administration of the shocking homologous antigen the animals were injected with solutions of an azocompound made by coupling resorcinol with diazotized *d*- and *l*-*p*-aminotartranilic acids. The products are designated as *d*-T.R. and *l*-T.R., respectively.

These substances were prepared as follows: 480 mg. *d*- or *l*-*p*-aminotartranilic acid were diazotized in the usual way (7, page 410) and coupled with 110 mg. resorcinol. The dye formed was precipitated with the aid of dilute hydrochloric acid, the precipitate washed in acidulated water and dried.

A 1 per cent solution of the dye was made in a 0.9 per cent salt solution by adding dilute sodium hydroxide, and the solution was adjusted to neutrality or faint alkalinity. 1 cc. of various dilutions was injected intravenously. The shocking antigen was injected in a quantity of 7 mg. (1 cc. of a 0.7 per cent dilution) which corresponds to 5 to 10 minimal lethal doses (see Table I). At the time of the tests the weight of the guinea pigs was about 400 gm.

The experiments (Table VI) demonstrate that with one exception in which the animal showed severe anaphylactic symptoms, the guinea pigs previously injected with the heterologous azodye died in typical

TABLE VI

Inhibition by the Injection of Azodyes

Animals Sensitized with d-Antigen

Guinea pig No.	*d*-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of *d*-antigen	Subsequent change in body temperature	Guinea pig No.	*l*-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of *d*-antigen	Subsequent change in body temperature
				°*C*.					°*C*.
67	10	1	Weakness	−4.5	77	10	1	† 4 min.	
68	10	18	Cough,	−1.1	78	10	18	† 4 "	
			slight		79	5	1	† 4 "	
			weakness		80	5	2½	† 4 "	
69	10	18	Dyspnea	−1.2	81	5	2½	† 5 "	
			spasms		82	5	3½	† 4 "	
70	5	1½	Weakness	−0.2	83	5	24	† 5 "	
71	5	2	Negative	+0.7	84	2.5	1	† 5 "	
72	5	2½	Slight	−0.7	85	2.5	2½	Few con-	−3.6
73	5	2½	Negative	−0.3				vulsions,	
74	5	24	Cough	−4.0				very sick	
75			Weakness						
75	2.5	1	Somewhat	−1.3					
			sick, weak-						
			ness						
76	2.5	2	Negative	+0.6					

Animals Sensitized with l-Antigen

Guinea pig No.	*d*-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of *l*-antigen	Guinea pig No.	*l*-T.R. mg.	Interval between injections in hours	Result and symptoms after injection of *l*-antigen	Subsequent change in temperature
								°*C*.
86	10	16	† 4 min.	96	10	16	Slight, weak	−2.8
87	5	2½	† 4 "	97	10	16	Negative	−1.0
88	5	3	† 4 "	98	5	2½	Negative	−0.2
89	5	3	† 4 "	99	5	3	† 5 min.	
90	5	3	† 5 "	100	5	3	Somewhat	−1.0
91	5	3	† 2 "				weak	
92	5	3	† 5 "	101	5	3	Weakness	−1.9
93	5	3	† 4 "	102	5	3	Slight, weak	−0.5
94	5	3½	† 4 "	103	5	3	Weak	−1.8
95	5	4	† 6 "	104	5	3	Negative	−0.4
				105	5	3½	Slight, weak	−1.6
				106	5	3½	Slight, weak	−1.6

acute shock, whilst all animals but one, injected with quantities of 2.5 to 10 mg. of the homologous dye, survived. The surviving animals exhibited but rarely typical anaphylactic symptoms as spasms or cough, although many appeared sick. With higher doses of the dye the protection was still evident even when the injection of the antigen was made the following day.

Another batch of animals was sensitized passively by injecting a potent precipitating immune serum produced in a rabbit by immunization with azoprotein made from horse serum and diazotized *p*-arsanilic acid.[2] On injecting intraperitoneally guinea pigs weighing about 300 gm. with 0.3 cc. of this immune serum, the animals proved to be sensitive to an azoprotein prepared from diazotized *p*-arsanilic acid and chicken serum. The minimal lethal dose was regularly found to be 0.5 mg. In the tests presented in Table VII the animals were passively sensitized with 0.3 cc. of the immune serum. The substance tested for inhibition was a product of coupling diazotized *p*-arsanilic acid and tyrosine (2). This was injected intravenously in a volume of 0.5 cc. at stated intervals prior to the administration of the antigen or in a mixture with antigen (indicated as "0" in Table VII). The antigen was employed in a quantity corresponding to two minimal lethal doses.

From Table VII it is seen that the animals were protected from lethal shock by quantities of 2.5 to 1.25 mg. of the dye regardless of the time interval between the two injections. These animals showed either no symptoms or became somewhat weak. In two animals only were slight convulsions or coughing observed. With 0.6 and 0.3 mg. of the dye the results were irregular but again the outcome appeared not to depend on the time elapsed after the injection. A still smaller dose (0.15 mg.) failed to prevent shock also when the dye was injected simultaneously with the antigen.

A few experiments with solutions of sodium *p*-arsanilate seemed to indicate that also this substance has an inhibitory effect upon the anaphylactic reaction but considerably larger quantities were used than of the azosubstance.

The protection described can be explained in two ways, either by assuming that this effect is similar to the inhibition of the precipitin reaction *in vitro* by simple substances containing the specific group (16), or that the mechanism is analogous to the well known desensitiza-

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[2] For the method see (15).

TABLE VII

Inhibition by the Injection of Azodye

Animals passively sensitized with 0.3 cc. immune serum; the next day injection of the compound of *p*-arsanilic acid and tyrosin, followed by the administration of 1 mg. of the shocking antigen.

Guinea pig No.	Atoxyl-tyrosine in mg.	Interval between injection of dye and antigen in hours	Shocking antigen	Result and symptoms after injection of shocking antigen	Subsequent change in body temperature
					°C.
107	2.5	3	1 mg.	Somewhat weak	−1.6
108	2.5	18	1 “	Few coughs, somewhat weak	−1.6
109	2.5	18	1 “	Negative	+0.55
110	1.25	0	1 “	Vigorous scratching	−1.9
111	1.25	0	1 “	Negative	−1.55
112	1.25	3	1 “	“	−0.75
113	1.25	3	1 “	“	−1.8
114	1.25	18	1 “	Spasms	−2.4
115	0.6	0	1 “	† 4 min.	
116	0.6	0	1 “	Convulsions, weak	−2.3
117	0.6	3	1 “	Spasms	−1.55
118	0.6	3	1 “	Negative	−1.25
119	0.6	3	1 “	“	0
120	0.6	18	1 “	† 4 min.	
121	0.3	0	1 “	Somewhat weak	−1.1
122	0.3	0	1 “	† 4 min.	
123	0.3	3	1 “	Dyspnea, somewhat weak	−1.45
124	0.3	3	1 “	Severe	−8.85
125	0.3	3	1 “	† 6 min.	
126	0.3	4	1 “	Negative	−0.9
127	0.15	0	1 “	† 4 min.	
128	0.15	0	1 “	† 4 “	
129	0.15	3	1 “	Very severe, almost dying	−2.4
130			0.5 “	† 5 min.	
131			0.5 “	† 6 “	
132			0.5 “	† 3 “	
133			0.5 “	† 4 “	
134			0.5 “	† 4 “	
135			0.5 “	† 3 “	
136			0.25 “	Slight to moderate	−3.0

tion by small quantities of antigen. On the first assumption one would expect protection to diminish with the elimination of the inhibiting substance from the blood stream. Actually the elimination takes place rather quickly since soon after the injection the urine is distinctly colored. Further evidence was gained from an examination of the color of the serum of guinea pigs after intravenous injection of the dye and from an estimation of the dye in the serum by inhibition of the precipitin reaction. From the few tests made it appeared that a considerable part of the azodye (about half) was already eliminated within the first hour after the injection.

On the other hand protection was still demonstrable on the day following the administration of the dye, and furthermore, in the experiments of Table VII there was no noticeable difference in the results whether the inhibiting substance was injected simultaneously with the antigen or 3 hours afterwards. Consequently one can conclude that the effect is not due simply to the presence in the circulation of the substances tested but to a desensitizing action upon the tissues. This view is corroborated by the observation that frequently the injection of homologous azodye into sensitized animals was followed by a significant drop in temperature and in a number of cases even by anaphylactic shock (17), but in some series of experiments this result could not be duplicated.

It is possible, however, that there are other instances in which protection is brought about by the same mechanism as inhibition *in vitro*. This is suggested by the results of Tillett, Avery, and Goebel, who noticed that their glucosides prevented shock only when injected just prior to the antigen, but not after an interval of 2 hours. The apparent discrepancy between their results and the present ones can probably be attributed to differences in the chemical nature of the substances used.

SUMMARY

Experiments with azoproteins containing stereo-chemical isomeric groups of *d*- and *l*-tartaric acid showed well marked specificity of the anaphylactic reaction to these antigens, in conformity with the results of precipitin tests.

Shock in these animals could be prevented by injection of azodye containing the specific groups. This phenomenon is ascribed to a desensitization.

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SURVIVAL AND MULTIPLICATION OF THE VIRUS OF POLIOMYELITIS IN VITRO

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The subject of the artificial cultivation of filterable viruses has again brought into prominence the experiments of Flexner and Noguchi (1) and their followers with the so-called globoid bodies of poliomyelitis. The present paper deals with an attempt to repeat the results of the studies of Flexner and Noguchi and it is therefore desirable to present in brief form the results of the earlier work.

The original paper of Flexner and Noguchi (1) described the globoid bodies as minute, formed structures, cultivated by specially devised methods from tissues of the central nervous system from both human and experimental sources. The tissues were either fresh or glycerolated, unfiltered or filtered through Berkefeld V or N candles.

Methods of Cultivation.—The culture medium consisted essentially of human ascitic fluid to which had been added a fragment of sterile fresh tissue, usually rabbit's kidney. The liquid was covered with a deep layer of sterile paraffin oil and of the two sets of cultures, one was placed in an anaerobic jar and the other kept outside. All tubes were incubated at 37°C.

In the tubes kept outside the jar, it was observed after 5 days that about the fragments of tissue an opalescence appeared which could be gradually diffused through the tube by gentle shaking. After another period of 3 to 5 days, the opalescence extended into the upper portion of the medium, but at the expiration of 10 to 12 days the diffuse but slight opacity began to diminish, and irregular particles formed and slowly fell to the bottom of the tube. In control tubes, either no change was observed, or a slight granular precipitate covered the tissue; and, while in tubes in which growth had been noted the opalescence increased in extent, in the control series the precipitate gathered progressively about the tissue fragments. In the corresponding tubes kept in an anaerobic jar, a similar opalescent growth was seen, although it was slighter than that just described and required a somewhat longer time to reach its full development.

For the demonstration of colony formation, a semisolid medium was used which comprised ascitic fluid and sterile rabbit kidney, to which sufficient 2 per cent agar had been added to produce a solid mixture. This medium was not successful for initial growths, but after about the third generation of growth in fluid medium, transfer could be effected to the solid medium. In this, growth was noted after several days as a diffuse opalescence which first appeared about the tissue fragments and gradually aggregated into visible, minute colonies. Later, the opalescence rose in the medium until it reached about 3 cm. below the surface, the upper limit of growth being sharply demarcated.

In the original paper, mention was made of the fact that initial growth could be secured in a medium from which either rabbit kidney or ascitic fluid had been eliminated, the former being replaced by using a somewhat larger fragment of poliomyelitic brain or the latter by adding brain tissue extract or sheep serum water. Cultures could not be obtained in the absence of both kidney tissue and ascitic fluid, which might each have contained living cells; modified media were inferior generally to a mixture of the above two components.

The results obtained by the use of the substituted media indicate that growth was derived primarily from the inoculum and not from the kidney tissue or ascitic fluid. The original observation supporting this view is that relatively greater success was obtained when fragments rather than emulsions of poliomyelitic tissue were used. For it has been found that bacteria may be introduced from the outside into cultures of brain tissue from herpes-virus encephalitis (2) and from poliomyelitis (3) during the process of grinding the tissue in the preparation of emulsions.

Characteristics of the Globoid Body.—The globoid bodies were originally described as minute, globular structures, measuring from 0.15 to 0.3 micron in diameter, and arranged in pairs, chains, and small masses, according to the nature of the medium. In fluid medium, pairs and chains predominated; within the tissues of poliomyelitic patients or infected monkeys, only pairs or small masses were discernible, never chains. The reaction to Gram's stain was variable, that is, the bodies retained the stain feebly in earlier generations, but more intensely in older cultures. In peptone medium the organisms appeared to be larger and retained the Gram stain more firmly.

In initial cultures, considerable difficulty was encountered in demonstrating the bodies during the first 4 or 5 days. They could be detected more readily on the sixth or seventh day, and maximum growth was obtained from about the eighth to the twelfth day. After 3 or 4 weeks, however, the bodies became enlarged and irregularly stained, although very minute structures, barely visible, were still present. These altered cultures were filterable through Berkefeld filters, just as were the globoid organisms.

It would appear, therefore, from the original description in which the authors stressed the ability of the globoid bodies to form definite colonies and to show a distinctive morphology, that the bodies were unequivocally organisms.

Pathogenicity in Monkeys.—The original paper emphasized the point that only exceptional cultures possessed pathogenicity in the monkey. Furthermore, the limit beyond which the virus itself was considered not to be infective was that represented by a second transfer of the original virus in the culture medium.

Two sets of inoculation experiments were reported: one comprising fluid and solid cultures of globoid bodies derived from four human cases, and the other, cultures from the M.A. virus highly adapted by prolonged propagation in monkeys.

The mixed cultures of the human strains, in the third generation, were inoculated into a monkey, which developed characteristic experimental poliomyelitis. A second passage from this animal to two normal monkeys, and later, a third passage to a single *rhesus*, proved successful. From three of these four animals, cultures of the microorganisms were recovered.

The monkey strains of globoid bodies were successfully inoculated as follows: One monkey was injected with a mixed culture representing the third and fifth generations, and a second, third and fourth animal, with fifth to sixth generation cultures, all developed typical poliomyelitis. The globoid bodies were recovered in cultures made from the brains of all these animals. Furthermore, from the second and third *Macacus rhesus* successful passage was effected through two successive series of animals. In addition, a fifth monkey was inoculated with a mixed culture representing the eighteenth and twentieth generations. This animal died on the third day from a secondary infection but the inoculation of a filtrate of the site of inoculation in the brain into a normal *cynomolgus* was followed, after 13 days, by characteristic experimental poliomyelitis.

In one instance in the foregoing experiments, mention was made of the fact that characteristic experimental poliomyelitis was induced with a culture derived from an affected monkey which, in turn, had also been inoculated successfully with globoid bodies.

To sum up, the original report offered evidence of specific pathogenicity of globoid bodies even to the eighteenth or twentieth generation and of the successful recovery of a virulent culture from an animal injected with the globoid bodies themselves.

In a later article, Flexner, Noguchi, and Amoss (4) extended the studies on the globoid bodies and found that the microorganisms survived in the semisolid medium kept at room temperature or at 37°C. for over 13 months. A test for virulence showed these cultures to be pathogenic 6 months later, or 19 months after isolation. In this communication the authors also reported the preparation of "mass" culture medium and the employment of the prodigious number of globoid bodies so obtained for pathogenicity tests. The mass cultures were injected three or four times, since the earlier studies had revealed that only exceptional growths were active and that repeated injections of the ordinary virus (5) sometimes induced paralysis. Thus, one *rhesus* monkey was inoculated intraspinally four times at intervals of 6 to 11 days with mass cultures representing the fourth to thirteenth generations. 5 days after the last injection the monkey developed characteristic experimental poliomyelitis. Another monkey was injected intraperitoneally four times with the same material, and at the same intervals as in the preceding experiment, and typical poliomyelitis was induced. The nervous tissue of these two monkeys, glycerolated for 6 and 70 days respectively, proved infectious. The effect of the repeated injections is cumulative, for it was shown by a control test that the final, or fourth injection, by itself, failed to affect a normal animal.

The experiments described led to the inference that there is a wide fluctuation in pathogenic action of the globoid bodies, and only exceptional strains are infective. In addition, it was emphasized that the recovery of the bodies in culture from affected monkeys is made only with difficulty. Finally, it was pointed out that, by means of mass cultures, the bacterial nature of the globoid bodies is unequivocally established.

The next communication, by Amoss (6), again presented evidence to indicate the fact that the globoid bodies are microorganisms, and that after long cultivation *in vitro* they become saprophytic and grow more readily in a considerable variety of media. Moreover, Amoss reported the recovery of two additional, but nonpathogenic, cultures from monkeys with experimental poliomyelitis induced by human and M.A. virus. He emphasized in this paper a fact that had already been pointed out in the previous articles (1, 4), namely, that highly parasitic cultures are refractory to artificial cultivation.

Smillie (7) contributed a series of twenty-two cultures of globoid bodies in fluid and semisolid media obtained from the tissues of seven monkeys with experimental poliomyelitis. Of these, eight different strains were inoculated into corresponding *rhesus* monkeys. Five animals were unaffected but three, receiving fourth and fifth generation growths, showed some degree of paralysis after intracranial or intraspinal injection. These latter reactions could not, however, be definitely proved as poliomyelitic. Smillie also found that established cultures can survive in the icebox for months; that dye indicators inhibit their growth; that the preferable pH of the medium is at blood neutrality; that strict anaerobiosis is essential and that body fluids, especially ascitic fluid, are required for their proper development. Most noteworthy is the fact, as shown also by Amoss (6), that after having once become established and accustomed to artificial media, the globoid bodies grow more readily and may be more easily transferred.

The observers mentioned in the preceding paragraphs have therefore defined the characteristics of a true culture of globoid bodies. In view of the minute size of the microorganisms, emphasis was laid on the possible confusion which could arise in distinguishing them from granular precipitates in the culture medium. Hence the criteria of a culture should comprise the following principles: (a) characteristic growth in special, anaerobic, fluid medium; (b) definite colony formation; (c) distinctive growth in mass cultures, and (d) agreement with the other properties already mentioned in the foregoing résumé. Finally, (e) in respect to pathogenicity, attention has been called by the early investigators to the fact that only exceptional cultures show infectiousness and that the saprophytic strains are cultivable with difficulty while parasitic ones are even more refractory. On the other hand, definite experimental poliomyelitis has been induced in monkeys with the first, third, fourth, fifth, sixth, eighth, eighteenth, and twentieth generations of the globoid bodies. The eighteenth generation has been calculated to represent a dilution of $1:24^{17}$ of the original virus—considerably beyond the limits of its inherent infectivity. In one instance, a culture in semisolid medium, in its eighth generation and kept at 37°C. for 13 months, had not lost its infectiousness, whereas ordinary virus in a similar medium survives no longer than 20 to 30 days (4). Furthermore, the microorganism has been recultivated from the tissues of monkeys in which poliomyelitis was induced by the globoid bodies (in one series, from all of four cases) and such strains have, in turn, again produced the experimental disease.

With this definition of a culture of globoid bodies in mind, we can now compare the results of other investigators with those of Flexner and Noguchi (1, 4), of Amoss (6), and of Smillie (7).

In 1918, Heist, Solis-Cohen, and Kolmer (8) reported the isolation from human and monkey poliomyelitic material of four different strains of globoid bodies. These cultures agreed morphologically and culturally, in fluid, semisolid, and mass-culture medium, with the descriptions originally given (1, 6, 7). Furthermore, a definite distinction was made by Heist and Solis-Cohen (9, 10) between globoid bodies and ordinary streptococci—a finding also reported by Smillie (7) and by the prior investigators. Although the cultures of Heist and his co-workers were carried successfully through at least ten generations, no inoculation tests were made.

On the other hand, the results of the cultivation tests reported by Tsen (11) are not so clearcut. After "many trials" he succeeded in finding organisms which he believed to be similar to globoid bodies. But they could not be maintained for more than three generations, and hence were not inoculated into animals. Since only fluid medium was used, and the "organisms" therein disappeared rapidly, it is likely that Tsen may have confused granular precipitate with the globoid microorganisms.

Into this category of granulations may also be placed the so-called globoid body cultures obtained by Foster (12) from the nasopharyngeal secretions of common cold cases, and those of Bradford, Bashford, and Wilson (13). Olitsky and McCartney (14), in discussing the significance of Foster's cultures, pointed out how the error of confusing precipitates with minute organisms may arise and, furthermore, attempted to indicate the methods by which such misinterpretations may be eliminated. In the case of Bradford and his co-workers, their cultures of so-called "globoid bodies," obtained from patients with influenza, trench fever, war nephritis, and a number of other diseases, were later shown to consist of artefacts (Arkwright—15)—a view which the investigators themselves finally accepted.

In a study concerning the etiology of epidemic encephalitis lethargica, Loewe and Strauss (16), using the fluid and semisolid culture media already described (1), recovered what was believed to be a minute, globular organism from the brain, from nasopharyngeal mucous membrane tissue and washings, from spinal fluid, and from blood of cases of the affection. These investigators stated that their filterable organism resembled in morphology and cultural characteristics the globoid bodies, but that it possessed distinctly different pathogenic effects. They have not, however, reported the distinctive bacteria-like growth, as described by Amoss (6), in mass-culture medium. The fact, however, that colonies were obtained indicates the occurrence of bodies of microorganismal nature in their cultures, which, the authors believe, should be placed in the globoid body group.

EXPERIMENTAL

As indicated in the introductory paragraph, we undertook to repeat the earlier work on the cultivation of the globoid bodies and, especially, to secure cultures which possessed pathogenic properties.

For this purpose, we had access to abundant material taken from monkeys which had developed experimental poliomyelitis after the inoculation[1] of a highly active mixture of two virus strains—M.A. and K—(17, 18) which had been propagated for many years in monkeys. The animals, as soon as definitely paralyzed and before becoming moribund, were etherized and the brain and spinal cord were removed with sterile precautions. The tissues were generally free from secondary bacterial contamination, or bacteria derived, during exposure, from the air. Inoculation of media was immediately carried out, using fragments of brain or spinal cord, or a Berkefeld V filtrate of a 5 per cent physiological saline suspension of these tissues.

Cultivation

The culture medium was that of Noguchi, consisting of ascitic fluid and a fragment of sterile, fresh, rabbit kidney.

In the preparation of initial cultures, we attempted to repeat precisely the original method described by Flexner and Noguchi (1). The fragment of rabbit kidney was placed in a test tube measuring 1.5 x 20 cm. The inoculum, consisting of either a small fragment of brain or of spinal cord, or of 0.5 cc. of the filtrate of these tissues, was next added and then about 15 cc. of sterile ascitic fluid. Finally, about 2 cc. of sterile liquid albolene was added in such a way as to form a layer over the surface of the medium. One set of tubes of each series was placed in the Boëz anaerobic jar (19), and a duplicate set kept outside. The incubation was at 37°C.

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[1] All inoculations in the monkey were carried out under ether anesthesia.

A modified technique was also used: sufficient 1 per cent neutral cysteine hydrochloride solution was introduced into the ascitic fluid to give a final concentration of 1:2000. The cysteine medium was sealed with solid petrolatum instead of liquid albolene. We found, however, that this modification did not lead to an increase in the number of positive "cultures."

As a rule, about 50 tubes of medium were inoculated during each original attempt at cultivation: two-thirds of them were set up as first described, and one-third with the cysteine.

After 10 to 14 days, the tubes were removed from the thermostat and a small amount of the medium was withdrawn from the bottom of each tube with a sterile capillary pipette, for film preparations. These preparations were allowed to dry in the air and, after being fixed by heat, were stained for 3 minutes with well-ripened, alkaline, methylene blue.

All tubes found by microscopic examination to be contaminated with ordinary, familiar bacteria were discarded. On the other hand, each culture thought to contain material resembling globoid bodies was subplanted to six or eight tubes of Noguchi's fluid medium and upon two rabbit-blood, dextrose-agar plates. In the case of each subplant, one-half the number of tubes and plates was placed in an anaerobic jar and the other half kept outside. In addition numerous uninoculated control tubes of the medium, and inoculated control tubes of dextrose broth were included in each series of transfers.

No characteristic macroscopic changes were visible in the inoculated tubes at any time. The opalescence described by Flexner and Noguchi (1) as appearing around the kidney at the end of the fifth day was not detected but a distinct clouding, possibly due to autolysis, was noted at the end of 24 hours.

With regard to the formation of colonies, which other workers (Flexner and Noguchi—1; Flexner, Noguchi, and Amoss—4; Amoss—6; Smillie—7, and Heist, Solis-Cohen, and Kolmer—8) agree in emphasizing as characteristic of the globoid bodies, and as indicative of their microorganismal nature, we were unable to secure any evidence. This may perhaps have been due to the fact that we did not employ the semisolid medium of Noguchi. However, no colonies could be detected on the plates, incubated anaerobically, which were made from tubes showing suspected growth. Nor were we successful in obtaining growth in the special mass-culture medium (4), although no less than twelve attempts were made by this method.

In view of our having used different media in this part of the work, any comparison to be made between our results and those of the original investigators should be based on findings in fluid cultures.

To recapitulate: Efforts at cultivations were made with fragments of the brain or cord or with Berkefeld V filtrates of the nervous tissues of seven monkeys inoculated with poliomyelitic virus and suffering from experimental poliomyelitis. With these materials, 315 tubes were inoculated, thirty-six of which showed minute morphological particles or "bodies" suggesting microorganisms. The subplanting from these "positive" tubes was disappointing, owing to the failure to obtain secondary growths in later subplants or to failure due to contamination with ordinary bacteria. Thus, while all seven monkeys yielded minute particles suggesting growths in initial cultures, the "growths" derived from three animals failed after the first subplant, from one after the third, from one after the fourth, from one after the eighth, and from the last monkey after the eleventh subplant.

Morphology

The microscopic appearances to be described relate wholly to the fluid subplants and it should be recognized that because of the presence of the autolyzing kidney fragment and the resulting granular precipitate, it is just such cultures that are most subject to misinterpretations. Indeed, the distinction in early subplants between minute microorganisms and precipitate is so indefinite that we were led to apply the method of cataphoresis in order to attempt possible differentiation. We already knew that the poliomyelitic virus in nervous tissue suspensions migrates to the anode (20). Applying the same experimental procedures (21) to washed "cultures" in twenty-three tubes derived from six different "strains" in five separate tests, no conclusive evidence could be obtained, by this method, of differentiation of "microorganisms" from precipitate. For whatever picture was detected at the anode could be seen also at the cathode. On the other hand, control experiments with *Bacterium pneumosintes* and *Bacterium coli* were clearcut; there was active migration of both to the anode.*

Notwithstanding these facts, we were able to pick out thirty-six from 315 tubes in which the distinctions were sufficiently wide to suggest the possibility of microorganisms being present. The main points of such distinctions are as follows: In early subplants, the minute and "globoid bodies" were single, in pairs, or in short chains, the individual bodies being of a fairly uniform appearance. In older subplants, the bodies were more numerous and in part formed agglomerated masses. In remote subplants, they were somewhat larger. The bodies stained well with well-ripened alkaline methylene blue and, as a rule, did not retain Gram's stain. In stained specimens the "bodies" appeared to be raised above the background of amorphous material, cellular detritus, and indefinite particles and were not refractile. It should be mentioned that in practically all film preparations we observed well-preserved kidney tissue cells and leucocytes, the latter probably derived from the ascitic fluid.

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* In carrying out the cataphoresis experiments, we were aided by the effective cooperation of Dr. D. C. Hoffman of The Rockefeller Institute.

Power to Infect Monkeys

We pass now to the most significant part of our study, namely, the power of the "cultures" to infect and induce experimental poliomyelitis in monkeys. Indeed, in one series of subplants, derived from the brain and cord of a monkey experimentally infected with the virus described in Experiment 1, we were successful in producing experimental poliomyelitis in monkeys with the materials taken from "cultures" in the seventh, eighth, ninth, and tenth transfers. It should be noted that the seventh transfer represents a dilution of the original material cultivated of about 1.5×10^{-12}, and the tenth, of about 1.3×10^{-18}. The history of the monkey yielding the active material is as follows:

Experiment 1.—Macacus rhesus inoculated intracerebrally on February 8, 1929, with 1 cc. of the suspension of anodic material obtained by cataphoresis of brain and cord tissues from a monkey in the paralytic stage of the experimental disease (20). February 19, excitement, tremor, right facial paralysis, and ptosis were noted. February 20, ataxia, tremors, weakness of right arm and shoulder and both legs were observed. February 21, the animal was prostrate and unable to move its arms and legs. Etherized. The gross and histopathological examinations revealed characteristic lesions of experimental poliomyelitis.

Sixty-nine tubes were inoculated with the infected nervous tissue of this animal. Of the sixty-nine tubes, eight showed what appeared to be a minute, "globoid microorganism." These were subplanted to sixteen tubes of which, after 12 days' incubation, five were "positive." A third subplant into thirty tubes yielded sixteen; a fourth into forty-two tubes yielded nine; a fifth of forty-five, eleven; a sixth of sixty-six, twenty; a seventh of sixty, thirty; an eighth of forty-eight, thirty-seven; a ninth of thirty-two, four; and a tenth of thirty-two, five "positives." All the forty tubes of the eleventh subplant were contaminated v moulds and diphtheroid organisms. By the time the sixth subplant was reached, all the "positive" tubes had a common origin from one tube of the first transfer.

The medium is notably irregular and uncertain in its composition because of the variations in quality of the ascitic fluid and possibly of the kidney tissue. Noguchi (22) originally pointed out that certain samples of ascitic fluid were more suitable than others for cultivating spirochetes and other investigators have emphasized this factor as influencing cultures of globoid bodies (4, 6, 7). That uncontrolled fluctuating factors played a part in our results is indicated by the discrepancies arising in the several subplants. The irregularity in positive tubes is correlated with the number of "bodies" visible under the microscope: In the first three subplants they were few, from the fourth to the eighth subplant, many; with the seventh subplant they predominated, but in the ninth to eleventh they became very few and difficult to find.

Monkeys were inoculated with materials from the seventh, eighth, ninth, and tenth subplants of these "cultures." The material inoculated consisted of centrifuged sediment of the tubes, washed, as a rule, five times with physiological salt solution. In one instance (Experiment 2) to the washed sediment of the seventh subplant was added the unwashed sediment of the sixth and fourth subplants.

Experiment 2.—Macacus rhesus A. April 18, 1929, pooled material from the bottom of the positive tube in the sixth transfer of the culture obtained in Experiment 1 (hereafter designated as Bodies 1) was prepared and of this mixture 2 cc. were injected intracerebrally, 10 cc. were injected into the peritoneal cavity and 2 cc. into the spinal canal.

April 23, pooled material from the bottom of the positive tube in the fourth transfer of Bodies 1 was prepared, and of this mixture 2 cc. were injected intracerebrally, 9 cc. intraperitoneally, and 2 cc. intraspinally.

May 1, the material from the bottom of the positive tube of the seventh transfer was centrifuged at high speed for 15 minutes; the sediment was suspended in 3 cc. of physiological saline solution, and then centrifuged again. This was repeated three times. 1.75 cc. of the final suspension were injected into the left cerebral hemisphere.

May 11, tremor; right facial paralysis; ataxia; complete paralysis of left arm, and legs very weak.

May 12, prostrate. Etherized.

Autopsy.—Meninges normal. A very small area of softening was visible in the border of the right internal capsule, involving the lenticular nucleus. This site contained old blood and yellow pigment. The midbrain showed spotty translucent areas about 1 to 2 mm. in diameter. The cervical cord contained pink, translucent, soft areas in the region of the anterior horn cells.

Microscopical Examination.—Forebrain: A moderate mononuclear cellular reaction was present. Lateral to and partly involving the lenticular nucleus was a small area of softening containing red blood cells in various stages of degeneration. Around this area were many phagocytic microglia. Certain of the nerve cells showed varying degrees of degeneration and some of them were phagocytized.

Medulla: A slight mononuclear infiltration of the pia-arachnoid membrane was seen. Moderate perivascular infiltration was present. A certain amount of nerve cell degeneration with and without accompanying cellular reaction occurred.

Cord: A slight lymphocytic reaction was observed in the pia-arachnoid membrane, together with a marked perivascular mononuclear infiltration. In the cervical region there was almost complete destruction of both the anterior and posterior horn cells, with accompanying phagocytosis of the débris by microglia. The same reaction was present but was progressively less marked in the lower levels of the cord.

A 5 per cent saline Berkefeld N filtrate was prepared from portions of the cord and of this 1 cc. was injected intracerebrally into a monkey. This monkey developed typical experimental poliomyelitis within 6 days.

Cultivation experiments in Smith-Noguchi medium were carried out with fresh central nervous tissue from Monkey A. Seventy-one tubes were inoculated and after 14 days' incubation, five tubes contained bodies resembling those with which Monkey A had been inoculated.

Experiment 3.—Macacus rhesus B. May 1, 1929, 2.5 cc. of a saline suspension of washed materials from the bottom of the positive tube of the seventh transfer of Bodies 1 were injected into the left cerebral hemisphere.

May 6, slight tremor; ataxia; marked left facial paralysis; both arms practically completely paralyzed.

May 7, prostrate; facial paralysis increased. Etherized.

Autopsy.—At a site of inoculation there was a soft area 2 x 2 mm. containing a small amount of golden yellow pigment. No cyst formation or frank hemorrhage was observed. The region of the anterior horn cells in the spinal cord showed injection and slight hemorrhage. This was most marked in the cervical region.

Microscopical Examination.—Brain: Slight perivascular lymphocytic reaction in the region of the thalamus. Irregular nerve cell degeneration. The choroid plexus was infiltrated with lymphocytes. In the sub-arachnoid space was a diffuse lymphocytic and mononuclear cellular reaction.

Medulla: Foci of mononuclear cells, some surrounding degenerated nerve cells, were present in the region of the fourth ventricle. A marked perivascular mononuclear infiltration occurred throughout the medulla. All stages of nerve cell degeneration were seen.

Cervical cord: A moderate meningeal and perivascular reaction was observed. The neurons showed marked destruction and active neuronophagia.

Thoracic cord: The meninges were thickly infiltrated with lymphocytes. The cells of the anterior and posterior horns showed marked acidophilic degeneration, but their outline was preserved. The nerve cells were surrounded by a large number of polymorphonuclear and mononuclear leucocytes. The same changes were observed in the lumbar cord.

Intervertebral ganglia: There was a marked interstitial lymphocytic reaction and a moderate acidophilic degeneration of the nerve cells.

Five monkeys were inoculated with suspensions and filtrates prepared from the cord of Monkey B. Three of these control monkeys developed typical experimental poliomyelitis within 7 days; the remaining two were unaffected.

Cultivation experiments in Smith-Noguchi medium were made with fresh material from the brain and spinal cord of Animal B. In the second transfer bodies closely resembling those inoculated were isolated.

Experiment 4.—Macacus rhesus C. May 15, 1929, 1.5 cc. of a saline suspension of washed material from the bottom of the positive tubes of the eighth transfer of Bodies 1 were inoculated into the left cerebral hemisphere and 1.5 cc. of the same suspension were injected into the spinal canal.

May 21, tremors; slight ptosis of both upper eyelids; ataxia; partial paralysis of both arms and shoulders; legs very weak. Etherized.

Autopsy.—Meninges normal. Site of inoculation used for touch preparations. A definite small hemorrhage was seen in the striate body. The cervical cord revealed definite areas of congestion and hemorrhage in the region of the anterior horn cells. These changes were less marked in the lumbar and sacral cord.

Microscopical Examination.—Site of inoculation: No bodies were seen in touch preparations. Lateral to the anterior part of the striate body was a narrow zone of softening containing red blood cells, granular débris, and a moderate amount of pigment. About the periphery of this area was a large number of glial nuclei.

Cord: The anterior horn cells showed varying degrees of degeneration, some being slightly involved while others were completely destroyed. A diffuse cellular infiltration of polymorphonuclear and mononuclear leucocytes was present. This reaction became less marked in the lumbar and sacral regions.

Intervertebral ganglia: A diffuse interstitial lymphocytic infiltration was present and a moderate number of nerve cells showed partial or complete degeneration.

A 5 per cent saline Berkefeld N filtrate was prepared from the brain and cord of Monkey C. 1 cc. of this filtrate was injected into the left cerebral hemisphere of a normal control monkey. Within 8 days the animal developed typical experimental poliomyelitis.

Cultivation experiments in Smith-Noguchi medium were made with fresh tissue of the central nervous system from Monkey C. After 14 days' incubation, one of forty-seven inoculated tubes contained bodies similar to those with which the monkey had been inoculated.

Experiment 5.—Macacus rhesus D. May 31, 1929, 1.25 cc. of a washed saline suspension of the material from the bottom of the positive tubes of the ninth transfer of Bodies 1 were injected into the right and left cerebral hemispheres.

June 6, tremor; ataxia; both arms paralyzed; legs weak.

June 7, prostrate. Etherized.

Autopsy.—The meninges were slightly injected. The left site of inoculation showed an old hemorrhage with some yellow pigment and an area of softening extending into the internal capsule and the caudate nucleus. A similar picture was present in the right site of inoculation. Typical translucent, congested and hemorrhagic areas were visible in the medulla and in the region of the anterior horn in the cervical cord. No organisms were seen in preparations made from the sites of inoculation.

Microscopical Examination.—The site of inoculation showed a hemorrhage with hemosiderin pigment and was surrounded by phagocytic mononuclear cells. A slight diffuse increase in glial nuclei was present. Nearly all of the anterior horn cells in the cervical region had disappeared. Many foci of phagocytic mononuclear cells were present. A moderate perivascular lymphocytic reaction was observed.

A 5 per cent saline suspension was made of the cord of Monkey D. 0.5 cc. was inoculated into the left cerebral hemisphere of a normal, control monkey, which in the course of 7 days developed typical experimental poliomyelitis.

Cultivation experiments in Smith-Noguchi medium were made with the fresh central nervous tissue from Monkey D. None of the forty-eight tubes showed bodies similar to those with which Monkey D had been inoculated.

Experiment 6.—Macacus rhesus E. May 31, 1929, 1.25 cc. of a washed saline suspension of the material from four negative tubes of the ninth transfer of Bodies

1 were inoculated into the right and left cerebral hemisphere [illegible] terial contained no demonstrable bodies.

The monkey remained well and was discarded after 1 month's o[illegible]

Experiment 7.—Macacus rhesus F. June 14, 1929, 1.5 cc. of washed saline suspension of material from the bottom of five positive tubes of the tenth transfer of Bodies 1 were injected into the right and left cerebral hemispheres.

June 20, excited; tremors; right deltoid weak.

June 21, prostrate. Etherized.

Autopsy.—There was slight meningeal congestion. An area on the left side, greenish gray to red in color, was filled with tenacious, soft, elastic material which contained staphylococci. The area extended from the superior surface of the frontal lobe to a region near the internal capsule, 1 to 2 mm. wide, and spread out at the base to a pyramidal area about 4 mm. in diameter. On the right side a similar condition prevailed, although the area was not as large but more hemorrhagic and extended almost to the internal capsule. The fourth ventricle revealed the usual picture of poliomyelitis, but its edges were bulging and the glistening surface showed pink, turgid, tiny, translucent, somewhat hemorrhagic areas. The cervical cord exhibited the characteristic injection and translucence of the anterior horns, but in the dorsal and lumbar cord the pinkish tinge was not as prominent.

Microscopical Examination.—Forebrain: At the site of inoculation an elongated area was seen extending from near the cortex to the external surface of the lenticular nucleus. This area contained erythrocytes, polymorphonuclear and endothelial cells, and granular débris. A small amount of golden yellow pigment was present. The tissue forming the border of this area comprised a mass of phagocytic mononuclear cells and proliferated neuroglia; there was also perivascular infiltration by lymphocytes. Lymphocytes infiltrated the pia-arachnoid. In the medulla there were present occasional nerve cell degeneration and neuronophagia with a moderate perivascular infiltration.

Cord: Cervical region. Moderate lymphocytic infiltration of the meninges. Almost all neurons showed acidophilic degeneration and some were in the process of neuronophagia. The gray matter was diffusely infiltrated with lymphocytes and with occasional polymorphonuclear cells.

Thoracic region: Here the cord was the seat of an intense reaction. No normal appearing nerve cells were present, all being degenerated and fragmented, the subject of active neuronophagia. Very marked extra-adventitial infiltration was noted.

Lumbar region: Lesions of meningeal mononuclear infiltration; nerve cell degeneration; neuronophagia; perivascular infiltration with some diffuse lymphocytic and mononuclear cellular infiltration throughout the gray matter. In general, the reaction was less marked here than in the cervical or thoracic cord.

Intervertebral ganglia: Moderate diffuse lymphocytic and mononuclear cell infiltration; a few necrotic nerve cells in various stages of degeneration with some showing neuronophagia.

A 5 per cent saline suspension was made of the cord of Monkey F; 0.5 cc. was inoculated into the left cerebral hemisphere of a normal monkey, which developed typical experimental poliomyelitis after an incubation period of 7 days.

Cultivation experiments in Smith-Noguchi medium were made with fresh tissue of the central nervous sytem of Monkey F. Two of twenty-eight tubes showed bodies similar to those employed in the inoculation of Monkey F.

Experimental 8.—Macacus rhesus G. June 14, 1929, 1.5 cc. of a washed saline suspension of the material from five negative tubes, that is, free from "globoid bodies," of the tenth subplant of Bodies 1 were inoculated into the right and left cerebral hemispheres. The monkey remained well throughout the period of observation.

One observes from the preceding experiments that the inoculation in monkeys of a saline suspension of the washed sediment from Smith-Noguchi cultures of poliomyelitic tissue induced, in the seventh, eighth, ninth, and tenth transfers, the clinical syndrome and the pathological effects characteristic of experimental poliomyelitis. It is noteworthy that in the successful experiments, the washed material contained small bodies resembling the globoid bodies described by Flexner and Noguchi. Equally important is the fact that in parallel experiments with material derived from the ninth and tenth transfers, the washed sediment of tubes showing similar bodies was active in monkeys while that of tubes not containing the bodies was inactive.

SUMMARY AND DISCUSSION

The study here reported concerns attempts at bacteriological cultivations with fragments of brain or cord, or with Berkefeld V filtrates of the nervous tissues, from seven monkeys successfully inoculated with poliomyelitic virus. With these materials, 315 tubes were inoculated, of which thirty-six showed minute bodies resembling the globoid bodies described by Flexner and Noguchi. However, a study of subplants from these minute, morphological particles did not convince us that we had in hand actual cultures of the globoid bodies, or indeed of any living microorganism.

Nevertheless, when washed sediments from subplants of one of the strains, representing the seventh, eighth, ninth, and tenth transfers, were inoculated into monkeys, the clinical signs and pathological effects characteristic of experimental poliomyelitis could be induced. The virulence of the "cultures" could not be ascribed to carrying over the original material into these remote subplants since the seventh transfer represented a dilution of the original cultivated material to about 1.5×10^{-12}, and the tenth, to about 1.3×10^{-18} if one assume, as the transfer technic justifies, a thorough mixing of the contents of each tube. On the contrary, it appears as if the poliomyelitic virus had multiplied *in vitro*, and had increased as a consequence of being in a medium of a modified living tissue-cell culture. For in practically all specimens we observed many well-preserved kidney tissue cells and leucocytes, the latter probably derived from human ascitic fluid, a component of the Smith-Noguchi medium. In this connection, it should be mentioned that the several lots of ascitic fluid used in the cultivation tests were recently obtained from patients and employed from a week to a month after their collection.

There remains for consideration the problem of the selective pathogenicity of the "cultures:" only the material of those tubes of the ninth and tenth transfers which showed the "globoid bodies" proved pathogenic; those respective tubes of the same transfers which were free from the minute bodies but apparently identical in all other respects, were avirulent. It may be that the virus was adsorbed to the particular bodies which we have found in the "cultures" and which resemble closely the globoid bodies of Flexner and Noguchi. Further elaboration of this study would be necessary, however, before such an inference could be regarded as a definite hypothesis.

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STUDIES ON THE BACTERIOLOGY OF EPIDEMIC INFLUENZA[1]

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It is now six years since I first came here to demonstrate the work done by Dr. Gates and myself on the bacteriology of epidemic influenza. The presentation (1) was then made under the egis of Dr. Charles E. Simon, at the beginning of his series of courses on Filterable Viruses. The courses were notable not only because they were the first to be given on this subject in a special department of a school curriculum, but also because they were comprehensive and scholarly. One should pause to think of the debt which modern medicine owes to Dr. Simon, and to mourn the loss of his charming and inspiring presence.

I shall try to discuss, within the hour, the work on the bacteriology of influenza which has been done during the past few years, and consider the subject in the light of more recent experience. In the interest of a continuous presentation, it is desirable to restate briefly the results of earlier experiments.

EARLIER OBSERVATIONS

In face of the severe pandemic of influenza of 1918, doubt was cast on the significance of Pfeiffer's bacillus as the causal agent of the disease. Competent bacteriologists failed to find the microörganism in the blood or nasopharyngeal secretions of early cases with any degree of regularity, although this organism, as well as other ordinary bacteria, could frequently be recovered from the lungs of persons who had succumbed to a secondary pneumonia. Moreover, it was often found as a secondary invader in other unrelated diseases, such as tuberculosis, whooping cough, and measles; in respiratory infections other than influenza, and in normal persons during interepidemic and epidemic periods. Antibodies against the bacillus could be demonstrated only irregularly in recovered patients and serological tests revealed many distinct types in the majority of cases, although the rapid spread of the epidemic might lead one to suspect the incitant to be of one type. Furthermore, no clearcut evidence was adduced to show that the bacillus was specifically pathogenic for laboratory animals, and finally, inoculations with vaccines did not appear to be generally effective in diminishing the incidence of the disease.

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[1] De Lamar Lecture delivered before the Johns Hopkins University School of Hygiene and Public Health, February 18, 1930.

The unparalleled spread of the 1918 pandemic and the confusion that existed in respect to the causal agent of influenza led a number of investigators to turn to the study of its etiology. While Dr. Gates and I were at work upon this problem, several articles appeared which reported the successful transmission of the symptom-complex of influenza from man to man and from man to monkey by the use of filtered material. Among the many reports were, notably, those of Dujarric de la Rivière (2), Nicolle and Lebailly (3), Yamanouchi, Sakakami and Iwashima (4), and Bradford, Bashford and Wilson (5).

The experiments made by Dr. Gates and myself concerned chiefly the cultivation by special methods of the filtered nasopharyngeal washings obtained from early cases of epidemic influenza. As a ground-work for the cultivation tests, animal transmission experiments were first done with the object of inducing an infection of an influenzal character. The three outstanding characteristics of the human disease which could be used as criteria for interpreting successful animal transmission experiments were considered to be (*a*) the leucopenia, (*b*) the diminished resistance of the lungs which permitted invasion of different bacteria and so opened the way to pneumonia, and (*c*) the pathological changes chiefly edema, emphysema and hemorrhage found in the lungs of those who succumbed.

The first attempts at transmission of the infection from man to rabbits were made with the unfiltered saline solution washings of the nasopharynx of patients in the early hours of uncomplicated, epidemic influenza. It was expected that the different bacteria ordinarily found in the washings might be suppressed by animal passage and that the specific effects of an extraordinary microörganism might thereby be revealed. Later, filtrates of similar washings through Berkefeld "V" candles were employed. Intratracheal inoculations in rabbits of the unfiltered or filtered material were followed, one or two days later, by characteristic effects (6, 7). They comprised mainly a mild and transitory febrile reaction accompanied by leucopenia which endured for a few days, and from which the animal wholly recovered unless bacteria such as reside ordinarily in the nasopharynx were also injected into the lungs. While such bacteria by themselves do not, as a rule, produce lesions under these conditions of experiment, they were able to multiply in lungs affected by the injection of influenzal washings, and cause severe, and often fatal, pneumonias. On the other hand, when a rabbit was killed at the height of the mild and transitory uncomplicated reaction, the lungs often revealed an inflammation characterized chiefly by edema, emphysema and hemorrhage, but free from the fibrinous and cellular consolidation which is characteristic of pneumonia of bacterial origin.

During the first animal transmission experiments, cultivation tests were made with the filtered nasopharyngeal washings from patients early in the disease and with fragments of lungs, and filtrates thereof, obtained from rabbits injected with influenzal secretions (8, 9).

The special methods used for cultivation of the influenzal material comprised the Smith-Noguchi medium, which consisted of sterile, human ascitic fluid, to which was added a small fragment of fresh, sterile rabbit's kidney, all under a petrolatum seal. Later, there were added collodion sacs prepared in a particular manner (10). The Smith-Noguchi medium was enclosed in the collodion sac, surrounded by distilled water or physiological salt solution. The addition of petrolatum seals established anaerobic conditions throughout the system, and the nutritive and growth-promoting substances of the medium diffused through the collodion in sufficient quantities to support a luxuriant growth in the surrounding liquid. The confusing protein precipitate which formed around the tissue of the medium was confined in the sac. Rabbit blood agar plates incubated anaerobically offered still another means for cultivation, and finally, broth prepared with cultures of *Bacterium coli* (11). In the case of the latter medium, the coli organisms were grown in broth under a petrolatum seal until the bacterial cloud was barely visible. Then the bacteria were killed by heat. The resultant products of *Bacterium coli* served to supply growth-promoting factors and thus enriched the medium.

The Smith-Noguchi medium and the blood agar plates were used for primary isolations of microörganisms from filtered influenzal secretions or rabbit lungs; the sacs, for separation of protein precipitate from minute filter-passing organisms so as to permit their demonstration more clearly especially in sparse growths. The coli broth was employed for the preparation of antigen for serological tests. In the course of time, the technique as outlined for isolation of filter-passing microörganisms was changed only in one respect, namely, a different fluid was used for suspending the nasopharyngeal secretions. In the earliest experiments, physiological salt solution was employed; later, dextrose Ringer's solution, and finally, broth. That broth favors the filtration of bacteriophage was first demonstrated by Bronfenbrenner (12), and that it acts similarly in the case of filter-passing anaerobes of the nasopharynx was shown by Mills, Shibley and Dochez (13).

There was isolated from the filtered nasopharyngeal secretions of patients in the early hours of influenza, and from the lungs of rabbits intratracheally inoculated with these secretions, a hitherto undescribed organism, called *Bacterium* (later, *Dialister*) *pneumosintes*. This bacterium was recovered from similar sources during the pandemic of 1918–1919 (8) and during the recurrent waves of epidemic influenza in 1920 (8), 1922 (9), 1923 (14), and 1926 (15). Mention should be made of the fact that during inter-epidemic or epidemic periods the microörganism was not found in cultures of nasopharyngeal secretions of apparently healthy persons, or of patients suffering from non-influenzal affections. Nor was it found in the lungs of normal rabbits or those inoculated intratracheally with non-influenzal material.

At this point, attention should be called to the fact that *Bacterium pneumosintes* has not been cultivated from a large series of patients with common colds. Mills, Shibley and Dochez (13) have obtained similar results. They have made every effort to cultivate the organism except the use of animal transmission and have failed to recover it in their extensive work with normals and with common colds. It appears, therefore, that up to the present the microörganism has been found only in association with true epidemic influenza.

As originally isolated, *Bacterium pneumosintes* was a minute body, of regular and bacilloid form, with its length measuring from 0.15 to 0.3 micron (150 to 300 millimicrons), about 2 to 3 times its breadth. It was capable of passing Berkefeld "V" filters and of resisting the action of 50 per cent glycerol for months. It multiplied slowly and then only under strictly anaerobic conditions. The microörganism was consistently Gram-negative and could be stained, but not deeply, with the usual basic dyes. It formed on the surface of blood agar plates minute, round, convex, amorphous colonies having a colorless or grayish translucence. In media other than the Smith-Noguchi, somewhat longer forms of the organism have been noted but these have reverted to the original minute forms on transfer to the Smith-Noguchi medium. Later observations have shown that the characteristics just described remain constant over a period of many years—in one case 11 years —when the microörganisms are maintained in the original Smith-Noguchi medium. Furthermore, in this medium the strains require transfer only at long intervals, that is, from six months to a year, since it was found that the organisms remain viable therein at least three years without subplantation.

In early cultures, the *Bacterium pneumosintes* induced in rabbits the characteristic clinical and pathological effects which followed intratracheal injection of the nasopharyngeal secretions of influenza patients and which have already been described. But this pathogenicity was lost during artificial cultivation. However, the more recent experience of Boëz (16) may be of interest in this connection. Old cultures of *Bacterium pneumosintes* were injected intracerebrally in rabbits. They were killed 48 hours later during a febrile reaction. The microörganisms were recovered only from the lungs, and then in pure culture. The other internal organs were negative.

On the other hand, rabbits immunized with *Bacterium pneumosintes* cultures developed specific agglutinins, precipitins, bacteriotropins and complement-fixing bodies (17). A significant feature of the serological tests is the fact that all the strains tested had similar antigenic properties and reacted identically with the specific antibodies produced by any one of them; in other words, all strains behaved as if derived from a common source. Furthermore, the blood serum of patients who had recovered from clinical influenza agglutinated one or more strains of the microörganism, although the serum obtained during an epidemic period from a group of individuals apparently free from an attack of influenza showed similar agglutinins but in a smaller number of instances (15).

With regard to the relationship of *Bacterium pneumosintes* to the incitant of epidemic influenza, it was stated (1) in 1923 that "it has seemed wiser, ... merely to report the experimental facts, and to defer decision of the precise relation which *Bacterium pneumosintes* bears to epidemic influenza until further experience is obtained." And again, in 1926 (15), "a large accumulation of observational and experimental evidence must be sought wherever available, and pieced together as opportunities permit," in the effort to determine this relationship.

The Pneumosintes Group of Microörganisms

Bacterium pneumosintes is not the only anaerobic, filter-passing, Gram-negative microörganism found in the upper respiratory tract of man. From the nasopharyngeal secretions of patients suffering from various respiratory infections and of normal individuals, a considerable number of different bacteria of this group has been cultivated. Credit is due to Dr. Avery of the Hospital of The Rockefeller Institute for his discovery of the first of the groups (later designated as Group I) by means of cultures of filtered nasopharyngeal secretions on anaerobic blood agar plates. His finding was quickly followed by the isolation of two other groups by Dr. Gates and myself (Groups II and III) (9), and again shortly thereafter of still two others by me (Groups Ia and IIIa). In all, at the present time, the literature records more than 30 supposedly different groups of microörganisms of new species isolated from the upper respiratory tract, of which 6 were cultured at The Rockefeller Institute, and 11 additional ones were isolated by workers trained there (Branham (2) and Levinthal (9)).

It is not to be supposed that the different species of these microorganisms are limited to this number. Others may be found from time to time. Nor has the last word been said about the specificity of each one of the groups. It is likely that in a number of instances there is duplication. However this may be, the number of the different groups of anaerobic, filter-passing organisms isolated from the nasopharynx of man is comparable to that of the aerobic bacteria found in the same region. Further work is necessary to show the significance of each group of bacteria, but for the present they may be tabulated and described briefly as follows:

Groups of Gram-negative, Anaerobic Bacteria Found in Filtered Secretions from the Nasopharynx

Bacterium pneumosintes, already described.

Group I (Olitsky and Gates' classification). Vibrios of variable length, motile, showing sparse granular growths in Smith-Noguchi medium. Colonies are large, heaped in the centers, with flat edges, and non-hemolytic. The organisms are autoagglutinable (9).

Group Ia. Long, bacilloid organisms, non-motile, growing as a diffuse cloud in Smith-Noguchi medium. Colonies are raised, round, and translucent, with flat edges; they exhibit zones of brownish discoloration. Serological tests reveal their specificity and distinction from members of the other groups.

Group II. Bacilloid, pleomorphic organisms, non-motile, showing no obvious signs of growth in Smith-Noguchi medium; they merely vegetate therein. Plate colonies are translucent and small, and resemble those of *Bacterium pneumosintes*; they do not change the appearance of the medium. Serological tests reveal their specificity (9).

Group III. Short, vibrio-like, tenuous, monomorphic organisms, motile, vegetating only in Smith-Noguchi medium. Colonies are large, with raised, crater-like centers and flat, irregularly spreading edges; they do not change the appearance of the medium. The organisms are autoagglutinable (9).

Group IIIa. Bacilloid, short, tenuous, delicate organisms, some vibrio-like, faintly stained by the usual dyes, non-motile and growing very feebly in Smith-Noguchi medium. Colonies are small, granular, with slightly peaked centers and edges which are toothed; they do not change the appearance of the medium. Serological tests show their distinction from members of other groups (18).

The next two groups have been recovered by Branham (19) from the filtered nasopharyngeal washings of patients with influenza or common cold. They are as follows:

Group I (Branham's classification). This troup has the distinction of retaining Gram's stain. It consists of very minute organisms, indefinite in shape, occurring in clumps, and showing microscopic, round, raised, transparent colonies.

Group II. Gram-negative cocci, in diplo form but occasionally in short chains, showing a granular growth in Smith-Noguchi medium and producing hemolytic, round, smooth, shiny, raised, translucent colonies.

Another microörganism, isolated from the filtered nasopharyngeal secretions derived from influenza patients, and called *Bacillus granuliformis*, has been reported by Pavlović (20). This type has a close morphological resemblance to *Bacterium pneumosintes* and is therefore a Gram-negative, anaerobic, non-motile, bacilloid organism, which is stained with difficulty. As described by Pavlović, it is pathogenic for rabbits, inducing a transitory reaction of fever and, generally, leucopenia. The lungs of affected rabbits show also a hemorrhagic lesion, free from pneumonic consolidation, and the intratracheal inoculation of the microörganism may be followed by secondary pneumonias, if combined in action with other ordinary bacteria. It is of interest to note that 60 per cent of the patients studied, in the 1927 epidemic in Belgrade, yielded agglutinins in their sera against this bacillus. Serological tests, furthermore, revealed that antipneumosintes serum agglutinated, and gave positive complement-fixing reactions with *Bacillus granuliformis*. Pavlović, however, maintains that the latter is distinct from *Bacterium pneumosintes*.

In addition, there are recorded by Garrod (21) and by Williams and her collaborators (22) the incompletely classified groups of anaerobic organisms also cultured from filtered nasopharyngeal secretions of man. Garrod reports 2 types: Type I, minute, anaerobic, filterable, Gram-negative coccus, producing microscopic, transparent colonies which are hemispherical and have clear-cut margins. Type II, short, tenuous, Gram-negative bacilli, sometimes curved, producing microscopic, transparent colonies which are pleomorphic, some being flat, others slightly raised or conical, with either smooth or crenated edges.

The next series of microörganisms to be described comprises groups of Gram-negative, anaerobic bacteria found in the nasopharynx of man, the filterability of which is unknown because reports of this characteristic are lacking. The bacteria are tabulated and briefly described as follows:

Groups of Gram-Negative, Anaerobic Bacteria Found in the Nasopharynx of Man

The first series are those isolated by Levinthal (23). They include 9 different groups of which

Group I (Levinthal's classification), *Streptrothrix anaerobiontica*, comprises thick, thread-like, also short and long bacilli which grow as a diffuse cloud in Smith-Noguchi medium, producing gas when dextrose is added. Colonies are raised, whitish and mucoid.

Group II. Long, tenuous, thread-like organisms forming raised, whitish colonies which are hemolytic.

Group III Short, monomorphic rods with polar bodies. Colonies are grayish, large and transparent. (These are called *Pasteurella anaerobiontica.*)

Group IV. Bacilli of marked pleomorphism, like Type III Pfeiffer's bacilli (Levinthal's classification). Colonies are small, flat and colorless.

Group V. Coccobacilli resembling Type I Pfeiffer's bacilli, showing a profuse growth in Smith-Noguchi medium. Colonies are flat, shiny and reddish-gray.

Group VI. Bacilloid, pneumosintes-like organisms producing dew-drop colonies. Serological tests show their distinction from *Bacterium pneumosintes.*

Group VII. Tenuous, short and long bacilli, non-motile, which form small, whitish colonies having brownish centers.

Group VIII. Pleomorphic bacilli, labile to Gram's stain, non-motile, forming small, clear, colorless colonies.

Group IX (*Streptococcus parvulus*). Cocci in short chains which remain alive but do not grow in Smith-Noguchi medium and produce minute, clear, colorless, flat colonies.

In addition there are six groups isolated by Thomson (24).

Group I contains organisms regarded by Thomson as types of *Bacterium pneumosintes.* They were isolated from a severe case of Engadine fever (influenza?). They alter boiled blood agar to a cherry red color, and this is the distinctive difference between the organisms and *Bacterium pneumosintes.*

Group II. Gas-producing diplococci which resemble *Staphylococcus parvulus.*

Group III. Non-gas-producing diplococci, which do not grow in fluid medium, otherwise resembling *Bacterium pneumosintes.* These organisms were isolated from the sputum of an influenza patient.

Group IV. Bacilli with marked pleomorphism which are hemolytic and form flat, round colonies with depressed centers.

Group V. Oval diplococci which do not grow in fluid media. They form grayish-white, somewhat transparent, soft and moist colonies.

Group VI. Bacilli which do not grow well in fluid media, but form grayish-yellow, soft, moist colonies.

Under this classification may be included also the 4 groups of anaerobic cocci and 4 of bacilli isolated by Noble and Brainard (25) from nasopharyngeal secretions:

Coccus I (Noble and Brainard's classification). Cocci labile to Gram's stain, forming delayed hemolytic zones and fermenting dextrose and levulose. These cocci were recovered only from cases of common cold (as were those of the next 3 groups) and resemble the organisms previously described by Hall (26).

Coccus II. Cocci, Gram-positive, showing indifferent colonies on blood agar plates.

Coccus III. Gas-producing, Gram-positive cocci which are non-hemolytic and ferment dextrose and levulose.

Coccus IV. The organisms resemble those of Group III but differ from them by fermenting saccharose.

Bacillus II (Noble and Brainard's classification). Gram-negative, slender rods, slightly curved and actively motile, which form minute, delicate and translucent colonies. They were isolated from a healthy subject.

Bacillus III. Gram-negative rods, slightly curved with rounded ends, non-motile and forming coarsely granular colonies with scalloped edges. They were cultured from a case of chronic rhinitis.

Bacillus IV. Long, thread-like, Gram-negative rods, showing granules when stained, and forming indifferent colonies on blood agar plates. They were recovered from a patient with acute rhinitis.

Group of Gram-positive Bacilli. Pleomorphic cocco-bacilli, non-hemolytic, which were isolated from a patient with common cold.

Finally, there may be added 2 organisms, the discovery of which has antedated the work of Dr. Gates and myself. They are:

Bacillus rhinitis of Tunnicliff (27), a Gram-negative, anaerobic, curved or wavy, long rod, actively motile and growing in granular form in Smith-Noguchi medium. The colonies are small, round, and "dull" and later become brown. Tunnicliff believed that she could reproduce "rhinitis" in man by swabbing cultures intranasally. The source of this organism is the nasal mucus from patients suffering from acute coryza.

The second is the *Staphylococcus parvulus*, a gas-producing coccus, first isolated in 1898 by Veillon and Zuber (28), and subsequently found in the mouths of man and animals by Lewkowicz (29), who named it *Micrococcus gazogenes alcalescens anaerobius.* A type of this organism which is hemolytic but produces no gas in Smith-Noguchi medium has been described by Branham (30). Noble and Brainard (25) and others have found this coccus so frequently that it is generally regarded as part of the normal flora of the nasopharynx.

It is an impossible task to make a complete list of anaerobic organisms, obtained mainly from filtered nasopharyngeal secretions because investigations are being carried on at the present time, notably by Long at the Johns Hopkins Hospital, and the results are consequently indefinite. Yet the tabulation made above should suffice as a basis for further work until the future demonstrates the significance of each group of these bacteria. It may be said now that in upper respiratory infections, the organisms appear to increase in number, as is shown by our experiments and those of Branham (19) and Long (data not as yet published). In this connection it is desirable to report the results of cultivation experiments made by Long and myself on naso-pharyngeal secretions derived from patients with the upper respiratory infections prevailing in epidemic form in 1928–1929.

Results of Cultures in the 1928–1929 Epidemic of Upper Respiratory Infections

During this epidemic of an upper respiratory infection—a disease which some have considered as obscure but not influenza (31)—we have found difficulty in selection of cases when the standard for comparison was the characteristic picture of 1918–1920 patients. Of the cases called to our attention, many were obviously non-influenzal, as for example, common colds, sinusitis, pharyngitis, etc., but several were selected for study in which the symptoms were more characteristic of epidemic influenza. There were in all 15 of this kind. In 7 of the 15, the final diagnosis was uncertain in 4 cases; epidemic pneumonia in 2; and pharyngitis in the remaining one. In each of the 7 in which the diagnosis was not definitely influenza, leucopenia was absent: the white blood cell counts varied from over 8,000 to 14,000. Special anaerobic cultures, as described above, of the filtered nasopharyngeal secretions revealed no growth in 2, and of the pneumosintes group of organisms, Groups I, Ia, II (Olitsky and Gates' classification), and *Staphylococcus parvulus* in the others.

The remaining 8 patients showed normal or low white cell counts, in spite of febrile reactions and a symptom-complex which resembled that of epidemic influenza. The disease, however, was mild. None of these patients developed secondary pneumonia; all recovered promptly. Anaerobic cultures of the filtered nasopharnygeal secretions showed the predominant and prevailing organism to be of the Group IIIa of pneumosintes-like bacteria. Seven patients yielded this type. The microörganisms next in frequency of occurrence and appearing concurrently with IIIa bacteria, were those of Group I in 5 cases; II in 4 cases, and Ia in 2 instances.

It is to be noted, therefore, that in certain epidemics of acute infections of the upper respiratory tract, one of the group of pneumosintes-like organisms may be the predominant and prevailing bacterium to be found in the filtered nasopharyngeal secretions.

Dr. Long and I were engaged in the cultivation experiments just described during the period from October, 1928 to March, 1929. Dochez, Shibley and Mills (32) investigated a type of acute infection of the upper respirator tract of man, usually designated by the term "Common Cold," cases of which were occurring at about this time. With the filtered washings derived from patients suffering from this type of infection, Dochez and his coworkers could communicate to apes experimental common colds which resembled closely the human disease. Furthermore, no such reactions were obtained with the secretions from healthy persons, free from upper respiratory infections. From the filtrates in all positive experiments, the investigators have been able to cultivate a Gram-negative anaerobe of Group I of pneumosintes-like organisms. On the other hand, they have cultivated another type of the "group" microörganisms from 6 of 8 filtrates derived from the nasopharyngeal secretions of healthy persons. These results, to which reference will be made again later, also indicate that one type may prevail in filtered secretions, and, as has already been shown (33), that certain "group" organisms may be found in health. In general, the results of Dochez and his coworkers agree with the prior conclusions of Dr. McCartney and myself obtained from inoculation experiments in man (33).

The bacteriological observations recently reported by Falk and his coworkers (34) and based on cases of upper respiratory infections occurring during the epidemic of 1928–1929 have revived interest in the etiological significance of green-producing streptococci. The cocci have been previously implicated as the incitant of influenza by Mathers (35) in 1917, by Tunnicliff (36) in 1918, by Rosenow (37) in 1919, and by Thomson (38) in 1929, but as yet these prior investigations have not been generally accepted as pointing to a solution of the problem. On the other hand, Falk believes that he has added evidence in support of the etiological rôle played by the streptococci. The evidence consists mainly of the recovery of 2 groups of serologically dissimilar cocci—one pleomorphic and the other monomorphic—obtained from influenza material, which produce pathogenic effects in lower monkeys and in man, a specific toxin, and specific agglutinin response against them on the part of recovered patients.

The Present State of the Problem of Etiology

Although considerable effort has been expended on the study of the identification of the causal agent of epidemic influenza, the present time finds the problem still unsolved. There are several factors which contribute to the existing confusion, and the outstanding difficulty arises from the necessity of selecting cases of undoubted acute influenza. There has been in the past frequent failure to recognize influenza as a specific, primary disease, and therefore to consider the various bacterial pneumonias that develop in lungs injured by the influenzal agent as secondary infections. Moreover, the primary, uncomplicated disease is usually mild and transitory; so that unless the incitant is sought in the early hours, it may become masked, or even supplanted by associated organisms. Furthermore, in view of the rapid and extensive spread of influenza during pandemics, it becomes difficult to select for control observations, cases of perfectly healthy individuals who have never suffered from the disease. Finally, to add to the confusion, a clear clinical distinction has not always been made between primary, uncomplicated, epidemic influenza and many different types of upper respiratory infections, such as common colds, acute rhinitis, acute bronchitis, and other indefinite conditions, which may simulate a true influenzal attack.

Hence there are three different agents for consideration and for future study of their precise relation to the cause of epidemic influenza: (*a*) Pfeiffer's bacillus, or other ordinary bacteria which are normally inhabitants of the upper respiratory tract; (*b*) a true filterable or ultramicroscopic virus, and (*c*) *Bacterium pneumosintes* or the group of pneumosintes-like organisms.

In the case of Pfeiffer's bacillus, the opinion is widespread that it plays the rôle of a secondary invader for the reasons which have already been mentioned. Yet there are still a number of investigators who believe that it is the sole exciting cause of primary influenza. The more or less ubiquitous occurrence of the bacillus need not necessarily indicate that it is not the incitant of the disease, for some investigators hold that from time to time its invasiveness and virulence increase to the point at which the organism becomes capable of inducing epidemics (39). Furthermore, many types of the bacillus have been discovered (40) since the original work of Pfeiffer, whose investigations opened up for study the vast field of hemophilic bacteria. It is thought by some that only a special type of pfeiffer's bacillus is involved in the causation and wide dissemination of influenza, and failure to realize this has led to confusion in interpreting experimental results. Others believe that symbiosis with other organisms, such as streptococci or the individuals of the aerobic flora of the oro-nasopharynx, is necessary for the growth and increase in virulence of Pfeiffer's bacilli (41). An important contribution to the subject of specific pathogenicity of Pfeiffer's bacilli was made by Blake and Cecil in 1920 (42). They employed a culture ultimately derived from a case of influenzal pneumonia, which had lost its virulence by prolonged artificial cultivation. Its virulence was restored by 11 successive passages in the mouse followed by 13 successive intraperitoneal passages in the monkey. Nasal inoculation of monkeys with the altered culture induced an infection which Blake and Cecil regarded as essentially identical with influenza with respect to its clinical course, symptoms, and complications. Later, intratracheal inoculations of 10 monkeys with this organism induced in 7 a hemorrhagic bronchopneumonia similar to the spontaneous pneumonia ascribed to the action of Pfeiffer's bacilli. The investigators infer, but do not definitely conclude, from the experimental results that the bacillus is the cause of influenza. But Jordan (39) explains the results by regarding the organism as a common secondary invader, inducing its characteristic effects which, in turn, obscure those of the primary incitant. Additional study is, however, necessary to show whether or not this, and the other beliefs mentioned are valid.

There are still others who assert that should the incitant of influenza prove eventually to be filterable, then Pfeiffer's bacillus need not necessarily be excluded from playing the rôle of causal agent, since it, also, is filterable. Pfeiffer, Prausnitz, Happe, and Dujarric de la Rivière (43) have shown experimentally that either granules in association with the bacillus, or the microörganism itself, might traverse the walls of the more porous Berkefeld and Chamberland filters (L1 and L2). Recent studies have proved that a distinction should be made between organisms which may traverse the more porous filters in numbers sufficient only to inoculate the filtrate and those which reach the filtrate in almost undiminished concentration (Mudd (44)). For example, Bronfenbrenner and Muckenfuss have reported (45) that instead of representing a filterable stage in the life cycle of the bacteria which sometimes yield growths in filtrates, the secondary growths are the results of imperfection of the filter itself, or of faulty technique. Grinnell (46) has summarized his views on the subject as follows: " . . . . the reported filtration of such organisms as streptococci, bacilli of the enteric group, diphtheroid bacilli and tubercle bacilli might be due rather to the passage of fragments of the bacteria in old cultures still capable of reproduction or to filtration in a particularly favorable suspension fluid than to the existence of a filterable stage in the life cycle of the organism." Hence in respect to its irregular filterability, Pfeiffer's bacillus appears to be no exception to the rule governing ordinary bacteria. In this connection the pneumosintes-like organisms are of a different order. They are ordinarily obtained from filtrates of nasopharyngeal secretions which are, as shown by cultivation tests, free from familiar bacteria, including, as a rule, Pfeiffer's bacilli. Mudd (44) believes that minute size or motility, characteristic of many of the pneumosintes-like organisms, is one of the important factors in effecting filtration.

Considerable attention has recently been given to the etiological significance of other ordinary bacteria of the aerobic flora normally inhabiting the upper respiratory tract. Of particular interest is the green-producing streptococcus. The fact that this organism ordinarily leads a saprophytic existence in the upper respiratory tract, that it is widespread in occurrence without approved relationship to health or disease, and that it is so ubiquitous as to make its recovery even from the air a relatively simple matter (Olitsky and Long (47, 48)), would indicate that its real significance as the incitant is questionable. But here again the problem may eventually resolve itself, as in the case of Pfeiffer's bacillus, in the implication of a particular, specific, pathogenic type. Since work on this subject is still in progress, judgment should be suspended until further proof is adduced.

With respect to the second class of possible incitants of epidemic influenza, namely, filterable or ultramicroscopic viruses, as distinct from cultivable, filter-passing microörganisms, it may be said that practically all of the experiments (2–5, 49–54) dealt with the transfer of the infection from man to man or from man to monkeys by means of filtered secretions. Although the transmission experiments in these instances were successful, cultivation tests either were not done or were made in a desultory manner so that it is not clear at the present time whether the particular experimental findings support the belief either of a filter-passing bacterium or of a true, non-cultivable, ultramicroscopic virus as the respective incitants. It is therefore apparent that here again additional experimental evidence is necessary before a conclusion may be reached.

The third class of possible incitants of epidemic influenza comprise the pneumosintes type of filter-passing microörganisms. Dr. Gates and I still maintain our cautious attitude in respect to the precise relationship of these microörganisms to epidemic influenza. Those who realize the difficulties of the problem will support this viewpoint.

To be sure, it may be said in favor of Bacterium pneumosintes that it has been recovered thus far only from influenzal material. Apart from our findings, the organism has been obtained by Loewe and Zeman (55), in New York, from the filtered nasopharyngeal secretions of influenzal patients, and has produced a characteristic clinical and pathological picture when injected into experimental animals (56). Gordon (57), in London, also reported evidence of the same bacterium in 14 of 20 influenza patients, and in 2 of 3 fatal cases. Lister (58), in South Africa, obtained 5 cultures of an identical anaerobe in 11 patients within 24 hours of the onset of epidemic influenza. He reported 4 febrile reactions, 1 fever and leucopenia, and 1 case of typical influenza among 12 volunteers sprayed with unheated cultures, and no reactions among 6 volunteers sprayed with heated cultures. In addition, Nakajima (59), in Tokio, cultivated 2 strains from pharyngeal secretions and 1 from the lungs of a fatal case of influenza. In the same year, Seitz (60), in Zurich, observed masses of minute bodies in the respiratory exudates of influenza patients and grew them for a time in mixed sputum cultures. Detweiler and Hodge (61) obtained 3 strains morphologically similar to *Bacterium pneumosintes* from filtered influenza material, 2 from lung filtrates and 1 from filtered nasopharyngeal secretions. Subcultures failed to grow, so that identification was not completed. The work of Thomson and the 3 strains which he has isolated from influenza cases have already been mentioned (24). In 1926, Hall (62) cultivated a strain of pneumosintes from the lungs of an animal experimentally infected with the secretions from a patient with typical epidemic influenza. Branham (19) reported the isolation of 4 cultures of either Group II or *Bacterium pneumosintes* from 3 patients with influenza and from an apparently healthy subject. These failed to grow after the first to seventh subculture, so that differentiation could not be completed. The cultivation experiments of Pavlović have already been mentioned (20). Finally, in 1929, Donadei (63) recovered from the nasopharyngeal secretions of a patient with influenza a pneumosintes-like organism which, before discernible cultivation in Smith-Noguchi medium could be obtained, had to be passed through the lungs of 5 successive rabbits.

In view of the uncertainty in identifying the different bacteria of the pneumosintes group, the question arises whether the microörganism reported by the investigators just mentioned have been definitely classified. This difficulty, and the discrepancy in the results of others who failed to cultivate any organisms of this type, show that the mode of procedure and the cultivation technique require further development and simplification.[2] After a simpler procedure and a more satisfactory medium have been devised, the problem of the exact relation of the pneumosintes organisms to the causal factor of epidemic influenza may be solved.

Common Cold and Epidemic Influenza

Before closing the subject, a brief consideration will be given to the relationship of common cold to epidemic influenza.

There has developed an opinion among several investigators, exemplified notably by Townsend (41), that an intimate correlation exists between the common cold and epidemic influenza. "Although we speak of influenza and the common cold (epidemic coryzas or catarrh, which may be taken to mean one and the same thing) as separate entities, it seems to be impossible for investigators who study the epidemiology of both conditions to separate conclusively influenza from the minor respiratory disorders, of which the common cold is a notorious example" (Townsend).

On the other hand, as Jordan (39) points out, epidemics of colds are related to weather and seasonal conditions, while pandemics of influenza are not notably so. Furthermore, the spread of colds is more limited in area and the rate of dissemination is slower; the age-specific mortality rates are different; the mortality from pneumonia and from abortion is specifically high in influenza. Finally, recovery from common colds does not leave a person resistant to a closely following attack of influenza (64).

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[2] In this connection it may be of interest to give the results of a recent experience. Old Smith-Noguchi cultures of influenzal material, taken in 1926, (15) and at that time set aside as negative, were recently (September, 1929) re-examined. A series of tubes from a common source, namely, filtered lung tissue from a rabbit inoculated with influenzal secretions, showed typical, identified, and now actively growing *B. pneumosintes*. In other words, the original cultures were too uncertain for identification, and furthermore, the pneumosintes organisms therein survived without transplantation for a period of at least 3 years. Also, an additional strain, missed in 1923, and freed ultimately from contamination over a period of 5 years, is now in stock. The 1923 report (14) should therefore read 5, instead of 4, pneumosintes cultures isolated—the added culture being derived directly from the secretions of an influenza patient.

A sharp clinical distinction can also be drawn between cases of true epidemic influenza and common colds. It is not necessary to describe the symptom-complexes here, since this has already been done in a comprehensive way by Jordan (39) and others. Attention, however, may be focussed on the well-known, and measurable, sign in influenza, namely, the leucopenia and especially, the early hours, the depression in mononuclear cells (65). This condition is absent in common colds, in which the white blood cell count may be normal, or higher than normal with a tendency to an increase in polymorphonuclear cells.

While the distinction between the two affections as they occur during pandemics or epidemics is sufficiently marked to permit definite diagnoses, no such characteristic differences may be revealed between severe common cold and mild influenza during inter-pandemic or inter-epidemic periods. It is such cases which add to the difficulty of the problem, as has already been pointed out. "But to identify as influenza all the sporadic cases, localized epidemics, and weather-conditioned outbreaks of clinically similar disease in inter-pandemic periods hardly seems justified" (Jordan (39)).

A wide variety of different incitants has been implicated as causal agents of the common cold (39, 41). The reports of Kruse (66) and of Foster (67) are of interest; these investigators have been able to produce colds in man experimentally with the filtered nasal secretions obtained from patients having the disease in an acute form. McCartney and I (33) have also obtained similar results in experiments in man and we have shown furthermore that while organisms of the pneumosintes group could be isolated from the spontaneous and the experimental disease, no one type could be, as far as our limited experience has shown, implicated as the causative factor. As mentioned earlier in this paper, however, one type of these microörganisms could be found prevalent and predominant in a group of patients studied at the same time. The results of the recent work of Dochez, Shibley and Mills (32) on anthropoids, already mentioned, are in accord with our findings on the transmissibility of common colds by filtered nasopharyngeal secretions and on the relation of the anaerobic, filter-passing flora to the natural and the experimental disease in man.

The inference which may therefore be drawn from the experiments with filtered secretions is that one type of upper respiratory infection, usually designated as common cold, may be caused by a filterable agent. Whether the agent is a true ultramicroscopic virus, or one of the filter-passing bacteria, remains to be determined.[3]

CONCLUSIONS

One may conclude from what has been stated in the preceding paragraphs that the question of the precise agent causing epidemic influenza cannot be, at the present time, definitely answered (68, 69).

The confusion which now exists may be ascribed to a number of factors. One obstacle to overcome is the difficulty of distinguishing between the primary, uncomplicated infection and the secondary pneumonias which develop in lungs previously injured by the true etiological agent; the lungs in such instances show the marked differences in histopathology resulting from the invasion of a wide variety of microörganisms. It should be mentioned that the different bacteria of ordinary species found associated with influenzal pneumonias are those which several investigators have at one time or another regarded as the primary cause of influenza. As compiled by Jordan (39), they comprise the *Bacillus pfeifferi*, pneumococci, green-producing or hemolytic streptococci, staphylococci, *Micrococcus catarrhalis*, members of the Pasteurella group, Friedländer's bacillus, and finally, combinations of two or more organisms of the ordinary species (the so-called composite, or symbiotic infections).

Another difficulty lies in the selection for study of a patient in the early hours of infection: the disease is transitory, the acute stage enduring for only a few days. As a rule, the patient comes to the attention of the bacteriologist when secondary invasion by ordinary bacteria has set in, which complicates, and perhaps suppresses, the primary incitant. Furthermore, epidemics occur explosively with the result that many cases may be offered for examination at about the same time and each cannot be studied in the most desirable way. Still another hindrance arises from perplexity in selecting, during widely spreading epidemics, individuals for control observation, that is, those who have not been attacked by the disease. Again, in respect to the subject of filter-passing anaerobes of the human nasopharynx, lack of a more simple, uniform and exact technique in collecting and cultivating material, and the absence of a precise taxonomy, are troublesome factors. Finally, experiments such as are made upon laboratory animals, including the lower monkeys, are hedged by impediments. Influenza is not known to occur naturally among them; hence the question arises when an experimental infection is induced as to whether it is or is not true influenza in the particular species involved. Many of these animals when taken from stock suffer from snuffles (70), as is the case with rabbits, or from this or obscure respiratory infections in the case of other animals.

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[3] The recent studies of van Loghem (van Loghem, J. J., An epidemiological contribution to the knowledge of the respiratory diseases, J. Hyg., 1928–1929, xxviii, 33) should be consulted for his hypothesis. He regards common colds as non-contagious, commensal infections induced by changes in air temperature; influenza, however, is contagious and follows its own epidemic course.

On the other hand, a survey of the recent work done on the subject of the incitant of epidemic influenza shows that in spite of all the difficulties which have been mentioned, considerable progress has been made. At the same time, the importance of influenza and related respiratory infections upon the communal well-being has been recognized with the result that the problem is receiving more attention by investigators than ever before.

The advances made in this field relate to sharper definitions in the clinical and pathological pictures of influenza and other upper respiratory infections and to the valuable epidemiological data of these affections. Furthermore, the large and important group of hemophilic bacteria, several members of which are definitely disease-producing, has been discovered, and the group of anaerobic filter-passing bacteria of the nasopharynx of man has been brought into light for examination for the first time. In addition, serious study is being given not only to the significance of each of the anaerobic and aerobic bacteria comprising the flora of the upper respiratory tract in influenza and related infections, but also to the problem of what constitutes the ordinary bacterial inhabitants of the naso-oropharynx in health. Finally, with regard to the selection of an animal suitable for transmission experiments, the recent work of Dochez and his colleagues has shown that the employment of anthropoids offers advantages which ordinary laboratory animals do not possess. It is hoped, therefore, that investigations in which apes are used as the animals of choice will make considerable progress toward the solution of the problem.

In the end, the expectation is that all obstacles will be eventually overcome and this in turn will lead to the goal of an exact knowledge of the etiological agent of epidemic influenza.

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A METHOD FOR SUPRAVITAL STAINING OF ANIMALS WITH NEUTRAL RED AND ITS PRESERVATION IN PARAFFIN SECTIONS

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The only method which satisfactorily preserves neutral red in sections of tissue supravitally stained is that reported by Cash (1)—or certain modifications of it—for the study of monocytes and epithelioid cells in the lungs of animals experimentally infected with tuberculosis.

Cash slowly injected into the ear vein 15 cc. of a 1 per cent solution of neutral red, dissolved in normal salt solution. In this manner he secured a true supravital staining of the connective tissue cells of the lungs. Any damage to the cells brings about diffuse staining of cytoplasm and nuclei and obscures those structures which are significant in the so-called supravital reaction. It is an essential part of the technique, therefore, to secure this reaction first. 10 minutes after the injection the rabbit was quickly killed. Thin slices of the lungs were fixed in Zenker-formol solution for from 12 to 24 hours. These blocks of tissue were quickly dehydrated, without washing, in acetone, cleared in benzene or xylol, and rapidly embedded in paraffin. Thin sections were then stained with various dyes, avoiding alcohols and water as much as possible. This method gave uniformly good results with lung tissue.

Prior to the experiments of Cash, McJunkin (2) reported a method for staining tissue supravitally and embedding it in paraffin. He exsanguinated animals from the carotids under light ether anesthesia and then injected saline solution, saturated with neutral red, directly into the tissues. The tissues were fixed in a Zenker formol solution. McJunkin states that the contact with the dye is irregular and that no observations can be made within the areas in which the cells are injured. Indeed, the weakness of the interstitial method of introducing the neutral red is the unevenness and uncertainty of obtaining a supravital reaction of the cells.

Gardner (3) states that the technique of McJunkin proved to be inapplicable for use on lung tissue and he therefore modified the methods of McJunkin and Cash. He injected the dye solution either intratracheally or intravenously. The fixation was carried out in the same fluid as that used by McJunkin, except that the formaldehyde was carefully adjusted to a pH of 7.6 with sodium hydroxide. Gardner claims that the sodium has a specific mordanting effect upon staining.

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It is well-known that the supravital technique of staining monocytes in the blood or tissues is the most reliable method we have for the accurate differentiation of these cells from other cytological elements. Good descriptions of it can be found in the papers of Simpson (4) and Sabin (5). Forkner (6) has recently described in some detail the methods, as used in Dr. Sabin's laboratory, of applying the technique to tissue cells. This description includes some minor modifications in the methods as originally reported by the above authors. Forkner (7) has shown that lymph nodes from certain areas of the body contain an abundance of monocytes, but that the exact relations of these cells in the tissues could not, by the methods then available, be demonstrated. The technique here recorded was elaborated for the study of this point. It has been found to be equally applicable for the study of all supravitally stainable cells throughout the body. The process of staining and rapid fixation was developed by the writer. The method of dehydration and embedding is a modification of that recorded by Cash (1), to whom I am indebted for suggestions in the development of this technique.

Method

Apparatus.—An instrument which has been of great value in this work is a modified, self-retaining cannula. It is made by inserting a fish-hook (Fig. 1, *a*), the curve of which has been eliminated by heating and straightening (Fig. 1, *b*), into the lumen of a No. 18 or 20 gauge transfusion needle in such manner that the barb of the hook protrudes over the edge of the needle, as shown in the accompanying figures (Fig. 1, *c*). About 1.5 or 2 cm. of the shaft of the hook lies within the lumen of the needle and if a slight bend is made in this shaft, it will retain its position within the lumen but can be removed for cleaning. When such a needle is inserted into the heart, or into a large vessel, the barb catches and prevents its coming out. In this way the labor, time, and trauma caused by the insertion and ligation of a cannula are avoided.

A large pressure flask (about 5 liters) is tightly stoppered and connected by means of rubber tubing with three outlets (Fig. 2) to (1) a blood pressure pump, (2) an Erlenmeyer flask containing 500 to 1000 cc. of 0.6 per cent neutral red (Grübler, *vital nach* Ehrlich) in 0.9 per cent sodium chloride solution, and (3) Erlenmeyer flask containing 500 cc. of Zenker's fixing fluid containing 15 per cent formalin and no acetic acid (Fig. 2). The flasks containing neutral red solution and the fixing fluid each has a glass tube extending to its bottom, in order that all the fluid can be pumped out when an increased pressure in the flask obtains. These glass tubes are connected by about 1.5 meters of small rubber tubing to two of the outlets of a three way stopcock. The remaining outlet of the stopcock is connected by a short rubber tube to the barbed cannula, as above described

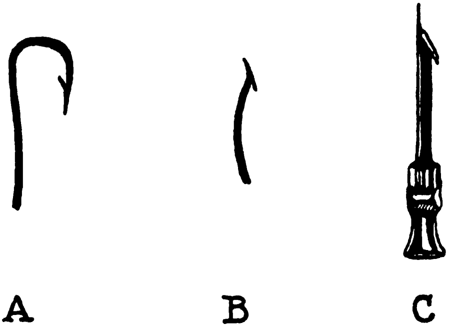

FIG. 1. Diagram illustrating construction of special cannula for perfusion of small animals through the heart.

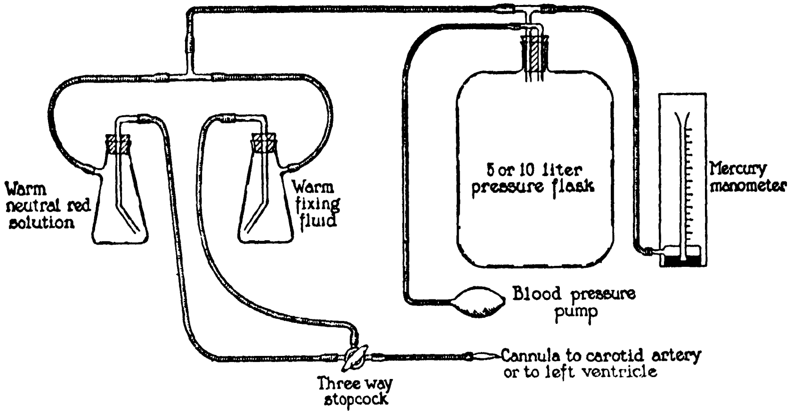
Warm neutral red solution
Warm fixing fluid
5 or 10 liter pressure flask
Mercury manometer
Blood pressure pump
Cannula to carotid artery or to left ventricle
Three way stopcock

FIG. 2. Apparatus used for staining of tissues and fixation of supravital dyes in cells of the entire body.

Staining of Tissues with Neutral Red.—An animal (rabbit, rat, guinea pig) is anesthetized with ether and fastened, ventral side up, with cords to an animal board. A jugular vein is exposed and ligated at the end proximal to the heart. An ordinary cannula is inserted into the distal segment. The animal should now be under deep anesthesia. Two routes may be used for injection of the neutral red. One can either insert a cannula into a common carotid artery, ligating the distal segment of the artery, and inject the neutral red through the carotid artery into the aorta, or a window about 2 cm. square can be quickly made in the lower left chest near the sternum, care being exercised not to injure the lungs. With a pair of blunt forceps, the parietal pericardium is torn away, the apex of the heart held, and the special self-retaining cannula quickly inserted into the left ventricle and the neutral red solution allowed to pass into the heart, under a pressure of from 100 to 150 mm. of mercury. It is understood, of course, that the injection system must be free of air. At the moment of injection, the cannula in the jugular vein is opened and the blood and neutral red solution are allowed to escape. The cords binding the legs are immediately released to promote a ready circulation through the entire vascular system. The perfusion proceeds for from 15 to 25 minutes, at the end of which time the entire animal is deep red in color. The perfusion fluid should be warmed to from 38° to 40°C. and the rubber tubing carrying the fluid immersed in a vessel of warm water at this temperature. If the heart becomes greatly distended, an incompetency of the mitral valve results with a back flow of neutral red into the lungs through the pulmonary veins and considerable fluid may thus escape through the trachea into the mouth. This may be avoided by clamping the trachea with a hemostat or by clamping off the left auricle. Likewise, intercostal or internal mammary arteries may be clamped off if excessive fluid is being lost.

The amounts of fluid described above are for rabbits. For animals of smaller or larger size, the amounts can be more or less proportionate. Considerably less neutral red solution is required if one injects by way of the carotid artery instead of through the left ventricle. In the rabbit or larger animals, the injection through the carotid is the method of choice. With rats, mice, and all small animals, the route through the left ventricle is much more feasible and it is for these smaller animals that the special barbed cannula is especially adapted.

Fixation of Neutral Red in Tissues.—After all of the neutral red has been passed through the circulation, the stopcock is adjusted so that the fixing fluid warmed to body temperature is injected immediately following the neutral red and through the same channels. About 500 cc. of fixing fluid perfused over a period of from 10 to 20 minutes will reach every organ. The cannulae and all instruments are then immersed in running water to prevent tarnishing with mercury. Instruments made of nickel-steel are not as readily injured by the fixing fluid. Organs are now removed and small blocks placed in a vessel or vessels containing the fixing fluid, where they are allowed to remain for from 12 to 24 hours at room temperature.

Dehydration and Embedding.—It is necessary that blocks be cut not over 2 mm. in thickness, in order that rapid dehydration can take place. Such blocks are dipped in tap water and blotted to remove the excess of fixing fluid. They are then carried through the following solutions, blotting gently after each step up to No. 7.

Solution	1.	Absolute ethyl alcohol—rinse for	1 minute
"	2.	" " "	5 minutes
"	3.	Pure acetone	20 "
"	4.	" "	20 "
"	5.	" "	20 "
"	6.	Xylol	20 "
"	7.	"	20 "
"	8.	56°C. Paraffin	1–2 hours
"	9.	56°C. "	1–2 "

The blocks are then embedded in 56°C. paraffin.

Staining.—The sections are cut from 4 to 10 microns in thickness, mounted on slides previously prepared with albumin fixative, and allowed to dry in a 37° incubator for 4 hours, or preferably over night. The sections on the slides can be excellently treated by the drop bottle method as follows: (1) Xylol to remove paraffin 10 to 20 seconds; (2) Absolute alcohol 5 seconds; (3) 95 per cent alcohol 3 seconds; (4) 95 per cent alcohol containing 1 per cent iodine 15 seconds; (5) 95 per cent alcohol several washings; (6) Absolute alcohol 5 seconds; (7) Clear in xylol; (8) Mount in balsam.

With the above method the macrophages, monocytes, and other vitally stained cells will stand out beautifully with the identical appearance of these cells, as seen in supravitally stained films of living cells.

An excellent counterstain can be employed after step (5) as follows:

(6 a) Distilled water for just long enough to overcome surface tension (3 to 5 seconds); (7 a) Goodpasture's acid polychrome methylene blue, full strength, for from 2 to 7 seconds; (8 a) Rinse quickly with water; (9 a) 95 per cent alcohol 5 seconds; (10 a) Absolute alcohol 5 seconds; (11 a) Xylol to clear; (12 a) Mount in balsam. Other methylene blue solutions may be used, but Goodpasture's permits of rapid staining, does not obscure the neutral red, and causes no troublesome precipitates.

Another method which is probably better than any other for demonstrating and preserving the cytoplasmic structures in supravitally stained macrophages and monocytes, and at the same time giving a satisfactory nuclear stain and a beautiful staining of the reticulum-fibers is a modification of Foot and Mènard's (8) method for staining reticulum by silver impregnation. The method is carried out in exactly the same manner as described by Foot, except that the sections are not bleached. When stained in this manner, the neutral red is of course washed out or obscured, but all the cytoplasmic structures which are stained with neutral red become intensely black. The monocytes, pre-monocytes, and macrophages show the typical characteristics, except that the cytoplasmic bodies are black. Detailed descriptions of these cells stained in the tissues by the above methods can be found in an accompanying paper (9).

SUMMARY

A simple, rapid method for staining all the supravitally stainable cells in the body, as in supravital preparations with neutral red, is described, together with a method for faithful preservation of the dye in paraffin sections. The essential points of the technique are, first, to secure the reaction of cells to neutral red which corresponds to the so-called supravital technique, involving the reaction of only those substances which respond to the dye while the cell is living; and second, to preserve the stain through the processes of fixation, embedding, and counterstaining.

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STUDIES ON BLOOD CYTOLOGY IN EXPERIMENTAL SYPHILIS OF THE RABBIT

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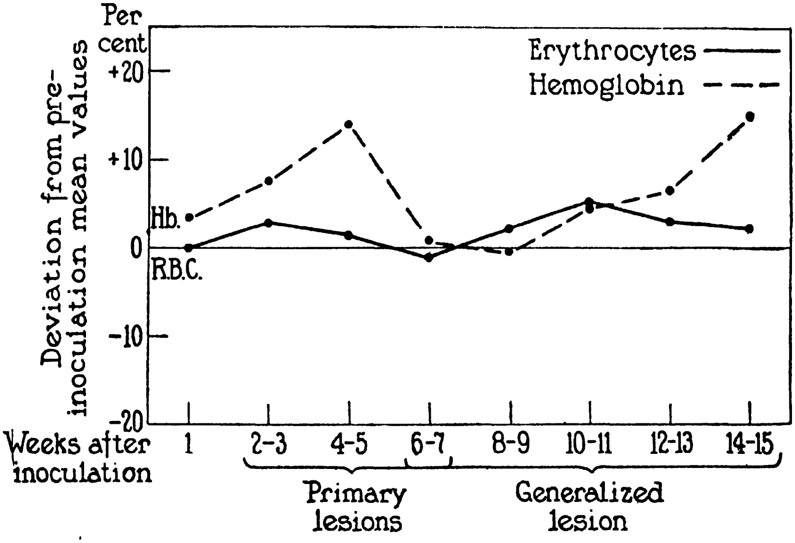
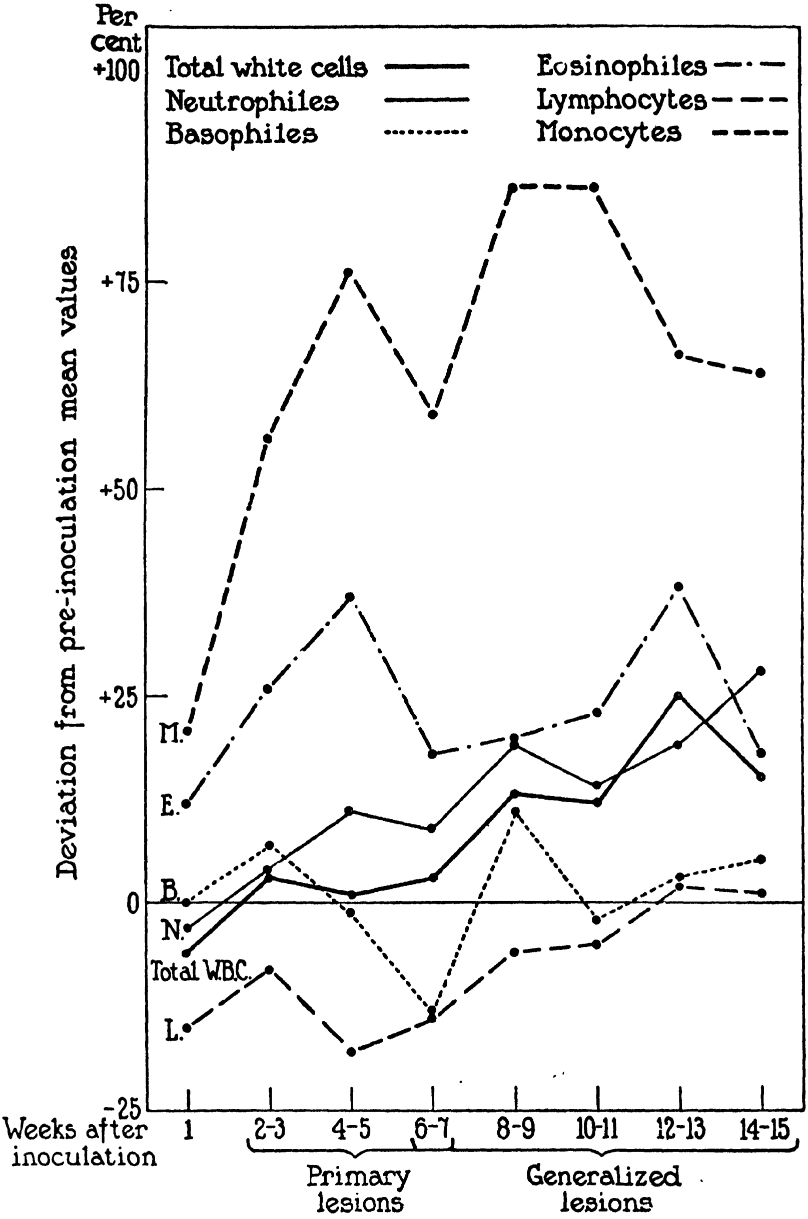
The character of the blood picture in human and experimental syphilis as determined by the newer methods of cytological study has received little attention. This is surprising in view of the considerable interest aroused by the results of such studies in other conditions. In tuberculosis, for example, recent investigations by means of the supravital technique in which the cells are observed in the living state, have led to modified and even to wholly different conceptions of the nature of the disease process. Although it may fairly be said that these new interpretations have as yet not received general acceptance, there can be no question that the observations themselves are of much significance in that they have been the starting point of new ideas and renewed discussion in a field of practical as well as of theoretical importance; namely, the nature of the defense reactions of the host under conditions of infection. In the case of syphilis, it was thought that the use of the supravital technique in the examination of both blood and tissues might furnish information which would aid to a clearer understanding of some of the problems presented by this disease.

According to most authors, a slight leucocytosis is usually found in the secondary stage of untreated lues, but as far as the differential white count is concerned, there is a diversity of opinion. Some writers have reported an increase in the proportion of the neutrophile cells while others refer to an increase of the lymphocytes. An eosinophila has also been reported, and attempts have been made to relate its degree to the intensity of the cutaneous eruption. In the primary stage, the white count is reported as being normal or slightly increased. Cases of tertiary syphilis, certain authors state, rarely show an increase of leucocytes while the differential count is not far from normal. On the other hand, a leucocytosis with a high lymphocytosis has been *described in connection with the not infrequent severe anemia of the* tertiary stage. It should be pointed out that the observations on the differential white cell count were obtained from dried blood film preparations stained by various dyes. As far as the red blood cells and the hemoglobin content are concerned, the general consensus of opinion appears to be that in the early stages of the disease, there is usually little change in the red cell count while in later stages there may be a more or less severe anemia, in which both the cells and the hemoglobin participate. Early in the course of the disease, however, before the cutaneous eruption has appeared, a marked reduction in the amount of hemoglobin has been noted by many observers.



TEXT-FIG. 1. Consecutive red cell and hemoglobin determinations. Combined group results expressed as mean percentage deviations from pre-inoculation mean values.

In connection with our investigations on experimental syphilis, especially with reference to the factors concerned in the reaction and defense processes of the host and the effects of environmental factors on these processes, a study of the blood by means of the supravital technique has been made. In addition, this method has been utilized in the examination of syphilitic tissue. The results obtained from the blood examinations will here be summarized.



TEXT-FIG. 2. Consecutive total white cell, neutrophile, basophile, eosinophile, lymphocyte, and monocyte determinations. Combined group results expressed as mean percentage deviations from pre-inoculation mean values.

In each of the five experiments under discussion, particular attention was paid to the clinical course of the disease, especially with regard to the incubation period of the primary and the metastatic testicular lesions, the location, extent, and persistence of generalized lesions, and the duration of the period of lesion activity as measured by the initiation of the condition of latency in which all disease manifestations have healed. Each experiment comprised a group of five to ten male rabbits, a total of 40 animals. The Nichols' strain of *Treponema pallidum* was used and all inoculations were made in one testicle with a saline suspension of syphilitic tissue containing actively motile spirochetes. The rabbits were observed three to five months after inoculation. Taking into consideration the various features of the infection just mentioned, the disease in these experiments may be described in general terms as of considerable severity in two and of a mild character in three groups.

Each blood examination included a total red and white cell count made with standardized pipettes, a hemoglobin estimation (Newcomer hemoglobinometer), and a differential white cell count in which one hundred cells were counted. The differential counts were made from supravital preparations stained with neutral red iodide. Before inoculation, each group received a variable number of examinations over a period of one to four weeks; after inoculation, the examinations were carried out at weekly intervals on the same day of the week. In each instance, the animals were examined at the same hour of the day and in the same order. All counts were made before feeding.

The results of this study have been analyzed for the present purpose of a general summary, on the basis of a combination of the results obtained in each group (Text-figs. 1 and 2). Such a method of analysis has the disadvantage of modifying individual findings, certain of which may be especially important but, on the other hand, it represents in general terms the particular character of the results. Absolute numbers of cells per cubic millimeter have been employed; the mean values of all counts for each animal before inoculation were used as a basis of comparison and the weekly post-inoculation counts have been expressed in the form of percentage deviations from pre-inoculation values. The group results for each week were obtained by determining the mean of the algebraic sum of the percentage deviations, and these group values were then combined in the same manner. Because of variations in the time reactions of individual animals with respect to the initiation, the development and the duration of disease manifestations, the results for each two weeks have been grouped together.

The findings with regard to the red cells showed that the counts were relatively unchanged or were slightly increased during the early stage of the disease, that is, during the period of active testicular lesions, and again during the height of activity of generalized manifestations. Subsequent examinations showed higher counts in certain cases. In the combined group results (Text-fig. 1), the curve representing the red cells shows a definite rise in these two periods.

An increased hemoglobin content of 10 to 15 per cent above pre-inoculation levels was a constant finding in each group during the early stages of the infection. In one group characterized by severe syphilis of long duration, the rise continued during the five months' observation period and reached a level of a 25 per cent increase. With a milder disease, a lowered hemoglobin content was usually found in the later stages, accompanying a rise of the red cell count. In the combined group results (Text-fig. 1), the early increase of hemoglobin is well illustrated.

A somewhat higher total white cell count, that is, 10 to 20 per cent above the pre-inoculation mean value, was generally found. In one group with mild syphilis, the level was practically unaltered. The increase occurred most frequently during the period of generalized manifestations, but in a group with severe syphilis, it was noted at the time of the development of the metastatic orchitis. The numbers of neutrophiles (psuedo-eosinophiles) were increased in a fashion similar to that of the total count, but the relative degree of change was usually slightly greater. These features are shown in the curves representing the combined group results (Text-fig. 2).

The eosinophile cells were also increased. Due to the small numbers of these cells in normal blood, the pre-inoculation mean values are more variable than is the case with the other cells, and any estimation based upon a count of one hundred cells involves an error of a similar greater magnitude. In each experiment, however, comparatively large numbers of eosinophiles were found after inoculation with

T. pallidum and not infrequently the level of a mean group increase reached 40 to 125 per cent above the pre-inoculation value. There was a definite tendency, moreover, for high counts to be found during the height of the testicular lesions and again during the active period of generalized lesions. The curve of the combined group results (Text-fig. 2) shows the general character of the change in the eosinophile counts.

The basophile cells of the several groups showed either comparatively slight numerical increases or somewhat diminished values. The combined results, as illustrated by the curve in Text-fig. 2, brings out the fact that an increase occurred early in the course of the disease and again during the period of generalized lesions. In both instances, this rise preceded that of the eosinophiles at corresponding periods.

It was found with respect to the lymphocytes that, in general, the number was not increased above the pre-inoculation mean and in certain groups, the count remained below this figure. In two groups, however, late in the course of the disease when the lesions were in process of regression and healing, an increase of 10 to 40 per cent above the pre-inoculation level as observed. The curve representing the combined lymphocyte findings for the several groups (Text-fig. 2) begins at a level well below the pre-inoculation mean and describes a fairly gradual rise to this value by the end of the observation period.

In the case of the monocytes, the marked numerical increase which was found in each experiment was very striking, and mean group values as high as 150 per cent above pre-inoculation levels were observed. The monocyte, which is easily differentiated by the supravital neutral red technique from the lymphocytes, and is now considered to be a distinct entity with a presumably individual physiological significance, is the cell which is usually classified as the transitional and the large mononuclear in the ordinary dried film preparation. With rabbit's blood, the number of monocytes is practically always smaller in a count made from a dried film preparation than in that made from a duplicate supravital specimen, due to the difficulties of identification and perhaps also to the destruction of a certain proportion of these cells. With human blood, the matter of identification is less perplexing.

In these experiments, a change in the numbers of monocytes was generally found by the time the diagnosis of a primary orchitis was made and increased values of approximately 80 per cent above pre-inoculation levels were observed. The condition of edema of the inoculated testicle which occurs in certain animals approximately three weeks after inoculation was usually associated with a marked rise in monocytes. With the initiation of a metastatic orchitis and of generalized manifestations, and during the period of activity of genital and generalized lesions, large numbers of monocytes were observed in the circulating blood. Regression and healing of lesions, on the other hand, was accompanied by diminished numbers. The combined group results (Text-fig. 2) show the general character of these findings.

A monocytosis has been observed in various diseases, as for example, tuberculosis, vaccinia, small pox, a malignant tumor of the rabbit, and virus III disease of the rabbit. It is reasonable to assume that some feature of the disease process common to these diverse conditions is associated with or responsible for the increased numbers of monocytes in the peripheral blood, and we have already suggested that the proliferative phase of the general reaction may be the feature in question (1).

Examinations of syphilitic tissue by means of the supravital technique, however, have shown few monocytes but large numbers of clasmatocytes. Similar observations have recently been reported by Morgan (2). It is possible that further studies of this character will yield information on the nature and function of these two cells, subjects which, at present, are under lively discussion.

This report represents a general summary of our completed observations on the blood picture in experimental syphilis. It is apparent that before final conclusions are drawn, the results must further be analyzed from the standpoint of the individual animal, taking into more precise account the time relations of the various phases of the disease. In addition, further observations under a variety of conditions should be made. At present, however, it may be said that in untreated experimental syphilis of the rabbit under the circumstances of these experiments, the outstanding features of the blood picture are: (1) a slight increase in the number of red cells and a somewhat greater increase in the hemoglobin content; (2) a slight increase in the total white cell count; (3) a slight increase in the number of neutrophiles; (4) a marked increase in the number of eosinophiles; (5) unchanged or lowered lymphocyte values in the earlier phase of the disease with increased values during the period of regression and healing of lesions; (6) a very marked increase in the number of monocytes throughout the period of disease activity.

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BACTERIUM GRANULOSIS IN RELATION TO TRACHOMA: ITS RECOVERY FROM EXPERIMENTALLY INFECTED MONKEYS AND FROM HUMAN TRACHOMA

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PLATE 26

(Received for publication, June 13, 1930)

Since Noguchi's report in 1928 (1), reports have come from laboratories in various parts of the world (Stepanowa and Azarowa in Kharkov (2), Finnoff and Thygeson in Denver (3), Addario in Palermo (4), Kendall in Chicago (5), Tilden and Tyler (6) and Olitsky and Tyler (7) in New York, and Lumbroso in Tunis (8)) of the isolation of *Bacterium granulosis* from trachoma and the reproduction in monkeys of granulomatous lesions of the conjunctiva such as were originally secured by Noguchi. It has now become desirable to assemble as many strains as possible of both European and American origin, in order that a comparative study can be carried out. Since the cultures survive for many weeks, even months, on the semisolid leptospira medium at ordinary temperatures, when sealed in ampoules, such a study is entirely feasible.

Certain animals described in Noguchi's monograph are still alive, 2½ years after inoculation, and continue to show the granulomatous lesions. The lesions in the chimpanzee "Louisa," which reached their maximum development within a period of 8 months, have since remained stationary. Of the *Macacus rhesus*, No. 80 shows, at the end of 2½ years, complete healing, with scar tissue, in the left eye (Fig. 2), while the right lid still carries a few follicles. Two *Macacus rhesus* (Nos. 5 and 6) inoculated from *Macacus* 73 in February, 1928, continue to have pronounced lesions. In June, 1929, No. 5 showed a spontaneous exacerbation, with marked redness and lacrimation, the cornea becoming diffusely cloudy (Fig. 3).

The present report deals with the following experiments: (1) the further transmission (a) by animal passage, and (b) by inoculation of cultures, of the Albuquerque Strain 1, isolated by Noguchi from Indian trachoma in 1926; (2) the recovery of the organism from monkey lesions, in their early stages, and also after they had persisted for a year or longer; (3) the isolation of new strains from cases of Indian trachoma, and (4) comparative cultural studies of the old and new strains and viability tests of the bacterium. The isolation of new strains was made possible through the cooperation of Dr. F. I. Proctor and the U. S. Indian Service. For clinical assistance we are indebted to Drs. Polk Richards and J. F. Lane, of the Indian Service.

1. Experiments with Albuquerque Strain 1 (Noguchi)

(*a*) *Transmission by Animal Passage.*—

The transfer of the infection from monkey to monkey was accomplished by means of tissue suspensions, the entire affected tarsal conjunctiva being removed, under ether anesthesia, ground in a mortar with a minimum quantity of isotonic sodium chloride solution, and injected subconjunctivally and also applied to the scarified conjunctiva, as described by Noguchi.

The first passage from two monkeys injected with cultures by Noguchi in June, 1927, (Nos. 72 and 73) gave positive results in most of the animals inoculated (Table 1). The only exception was an experiment (9) in which the suspension from the right conjunctiva of No. 72 was distributed among 15 animals. This diluted material failed in all but one instance to produce infection. With later passages from the same series the incubation period became longer and the positive results less frequent, and further transmissions were abandoned in favor of experiments with cultures.

(*b*) *Transmission by Means of Cultures.*—Although the original cultures had been carried on artificial medium for a period of 2½ to 3 years, having been transplanted every 6 to 8 weeks, inoculation tests gave positive results (Fig. 4) in about one-third of the animals (Table 2), showing that *Bacterium granulosis* may retain its pathogenicity for long periods of artificial cultivation.

TABLE 1

Transmission of Albuquerque Strain 1 by Passage

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left*	Right	
	1927		*days*			
1	Dec. 9	Conj. susp. No. 72 N**	13	++++	++++	Lesions persisted 1½ years. Died
2	"	"	13	++++	+	Lesions persisted 1 year. Right eye negative until 11 months after inoculation
	1928					
3[1]	Feb. 10	Conj. susp. No. 73 N**		–	–	
4[1]	"	"		–	–	
5[1]	"	"	66	++++	++++	Lesions still present (2 years). Cultures recovered
6[1]	"	"	45	++	++	Receded in 1 month
7[2]	"	"		–	–	
8[2]	"	"	45	++++	++++	Lesions on right still present. Cultures recovered
9[2]	"	"	33	++++	++++	Lesions persisted 20 months. Cultures negative. Died
10[2]	"	"		–	–	
11	"	"	66	+++	++++	Lesions still present after 19 months. Cultures recovered. Discarded
12	"	"	33	++++	++++	Lesions persisted 16 months. Discarded
13	"	"	139	+++	+++	Lesions persisted 8 months. Discarded

* Inoculations are always made into the left conjunctiva under novocain anesthesia (2 per cent).

** Numbers followed by N refer to monkeys inoculated by Noguchi, and protocols will be found in his report.

[1] Received 5 subcutaneous injections of heat-killed cultures of Strain 1 Noguchi on Jan. 9, 13, 17, 24, and 31.

[2] Received 5 intravenous injections of cultures of Strain 1 Noguchi on Jan. 9, 13, 17, 24, and 31.

TABLE 1—*Concluded*

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left*	Right	
	1928		*days*			
14	Feb. 10	Conj. susp. No. 73 N**	66	+++	+++	Lesions persisted 14 months. Discarded
15	"	"		—	—	
16	Mar. 9	Conj. susp. No. 72 N		—	—	Reinoculated May 16, 1928
17	"	"		—	—	Died of tuberculosis May 16, 1928
18	"	"	24	++	+	Lesions persisted 15 months, but did not progress further
19	Apr. 13	Conj. susp. No. 2		—	—	
20	"	"		—	—	
21	"	"		—	—	
22	"	"		—	—	
16	May 16	Conj. susp. Nos. 86 N, 79 N, and No. 1	83	—	++++	Died of tuberculosis in 3 months
23	"	"		—	—	
24	"	"		—	—	
25	"	"		—	—	
26	"	"		—	—	
"June" chimpanzee	June 20	Conj. susp. "Venus" and 81 N	66	+++	++++	Lesions persisted 1 year; disappearing
31	"	"	48	—	++	Died in 4 months
32	"	"	132	+++	—	Lesions persisted 13 months and remained unilateral. Discarded
33	"	"		—	—	
34	"	"		—	—	

TABLE 2

Inoculations with Routine Subcultures of Bacterium granulosis (Strain 1 Noguchi)

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1928					
27	June 12	Cultures on horse blood and horse serum agar plus carbohydrates and on semisolid medium, 4 and 13 days old	16 days	++++	++++	Lesions persisted 6 months. Cultures negative at 176 days, transmission positive
28	"			–	–	Discarded after 140 days
29	"			–	–	"
30	"			–	–	"
"Paulina" chimpanzee	Aug. 8	Similar cultures, 4 days old	16 days	+	+	Few follicles at border of tarsus. Did not progress further
35	"	"		–	–	
36	"	"		–	–	
37	"	"		–	–	
38	"	"		–	–	
"Julia" chimpanzee	Nov. 21	Similar cultures, 4 and 15 days old	42 days	++	++	Few granules at border of tarsus, and on tarsal plate. Did not progress further
41	"	"		–	–	Discarded after 6 months
42	"	"		–	–	Discarded after 7 months
43	"	"	5 mos.	++	++	Lesions receded after 2 months
44	"	"		–	–	Discarded after 117 days
	1929					
61	Jan. 24	Similar cultures, 6 days old	11 days	++++	++++	Lesions progressed for 8 months. Cultures positive at 4 months. Few follicles remaining after 11 months. Discarded
62	"	"	11 days	++	++	Transient lesions, disappeared in 4 months
63	"	"	11 days	+++	+++	Died in 3 months

2. *Recovery of Bacterium granulosis (Albuquerque Strain 1) from the Monkey Lesions*

The material used for isolation experiments was similar to that employed for direct passage. The basic medium was 2 per cent nutrient agar, adjusted to pH 7.4, to each 100 cc. of which was added 12 cc. of defibrinated horse blood and 5 cc. of a Berkefeld V filtrate of a mixture of 10 per cent dextrose, 10 per cent saccharose, 2.5 per cent mannose, 2.5 per cent levulose, and 2.5 per cent inulin. The conjunctival suspensions were smeared over the freshly prepared plates, which were then strapped with adhesive tape and incubated at 28°C. for 3 to 6 days.

TABLE 3

Recovery of Bacterium granulosis from Monkey Lesions

Monkey No.	Inoculated		Incubation	Result		Date cultured	Result
	Date	Material		Left	Right		
	1927		*days*				
81 N	Oct. 13	Conj. susp. "Louisa"	89	++++	++++	Dec. 5, 1928 (390 days)	+
	1928						
5	Feb. 10	Conj. susp. 73 N	66	++++	++++	Dec. 5, 1928 (299 days)	+
27	June 12	Strain 1 cultures	16	++++	++	Dec. 5, 1928 (176 days)	–
8	Feb. 10	Conj. susp. 73 N	45	++++	++++	Jan. 31, 1929 (356 days)	+
12	"	"	33	+++	+++	Jan. 31, 1929 (356 days)	–
46	Dec. 5	Conj. susp. No. 5	47	+++	+++	Jan. 31, 1929 (57 days)	+
9	Feb. 10	Conj. susp. No. 73 N	33	++++	++++	Mar. 20, 1929 (405 days)	–
11	"	"	45	++++	+++	Mar. 20, 1929 (405 days)	+
18	Mar. 9	Conj. susp. No. 72 N	24	++	+	Mar. 20, 1929 (377 days)	–
	1929						
61	Jan. 24	Strain 1 cultures	11	++++	++++	Apr. 5, 1929 (71 days)	+
	1927						
Chimpanzee "Louisa"	May 20	Conj. susp. chimpanzee "Kitty" (3d passage)	28	++++	++++	May 8, 1930 (1083 days)	+

TABLE 4

Inoculation of Cultures Recovered from Monkeys

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1928					
55	Dec. 12	No. 81 N cultures, I gen., 52 hrs. old, semisolid medium		—	—	Discarded after 177 days
56	"		147 days	++++	++++	Lesions persisted 9 months
57	"			—	—	
58	"			—	—	
51	"	No. 5 cultures, I gen. 52 hrs. old, semisolid medium	100 days	++++	++++	Marked congestion and secretion noted 65th day of disease (Fig. 5). Cultures (secretion) negative
52	"	"		—	—	
53	"	"		—	—	
54	"	"		—	—	
	1929					
60	Jan. 16	No. 5 cultures, 12 days old, blood agar and semisolid	142 days	++++	++++	Lesions persisted 4 months. Still marked when animal was discarded
59	"	"		—	—	
64	Jan. 24	No. 5 cultures, 6 days old, blood agar and semisolid	11 days	+++	+++	Lesions progressed for 6 months
65	"	"	11 days	+++	+++	"
66	"	"	11 days	+++	+++	"
67	"	No. 81 N cultures 6 days, blood agar and semisolid	22 days	+++	+++	Lesions persisted 6 months
68	"		11 days	+	+	Died of pneumonia, 4 months
69	"	"	11 days	+++	+++	Receding after 7 months. Discarded
45	Apr. 3	No. 8 cultures I, II, III gen.		—	—	
47	"	"	35 days	+	±	Negative after 5 months
48	"	"		—	—	

TABLE 4—*Concluded*

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1929					
52	Apr. 3	No. 46 cultures I, II, III gen.	35 days	++++	++++	Lesions persisted 7 months
53	“	“		–	–	
54	“	“	65 days	++++	++++	Lesions persisted 7 months
70	“	No. 61 cultures III gen., blood agar and semisolid	1–2 mos.	++	–	Transient lesions
71	“	“	21 days	++++	++++	Lesions persisted 6 months. Discarded
72	“	“		–	–	
73	“	“	21 days	++++	++++	Lesions persisting (10 months)
74	May 17	No. 11 cultures blood agar and semisolid IV gen.		–	–	Discarded after 4 months
75	“	“		–	–	“
76	“	“		–	–	“
77	“	“		–	–	“

Cultures were recovered from 6 of 10 *Macacus rhesus* cultured, and from the chimpanzee “Louisa.” With one exception, these animals had all been infected by passage from other monkeys; one *rhesus* had received cultures. The periods elapsing between inoculation and culture varied from 57 days to nearly 3 years (1083 days), and the length of this period seems to have had no relation to the success of recovery. The recovery of the organism from the chimpanzee “Louisa” 3 years after inoculation shows that the organism may persist in the experimental infection, as in the human disease, for a long period of time.

The cultures recovered in some instances proved highly virulent (Fig. 5).

3. *Recovery of Bacterium granulosis from Human Trachoma Lesions*

(*a*) *Fort Defiance Cases.*—In May, 1929, an expedition was made to Fort Defiance, Arizona, where is situated one of the two special schools which the United States Government maintains for the segregation of Indian children having trachoma. Five children who had been receiving daily treatment with copper sulfate or silver nitrate and two with advanced trachoma who had never received any treatment were selected for study.

Case 1. K., female, probably between 7 and 9 years old. Has been in the school 1 year; had advanced trachoma when she came. Scar tissue present. Treated with silver nitrate daily from Sept. 1928 to Jan. 1929, since then with copper sulfate (until May 16). Tissue taken May 23, 1929.

Case 2. A. L., female, about 7 years old. Has been in the school 2 years. Had advanced trachoma when she came. Scar tissue present. Treatment as in Case 1. Tissue taken May 23, 1929.

Case 3. N. T. Y., male, about 8 years old. Has not been in the school and has had no treatment. Mostly scar tissue; few follicles along edge of tarsus. Portion of tissue showing follicles taken.

Case 4. S., male, 7 years old. Conjunctivae covered with follicles, not much scar tissue. Has not been in the school and has had no treatment. Duration of disease unknown.

Case 5. A. L., female, 12 years old. From St. Michael's school. Disease probably of 3 to 4 years duration. Upper lids show mostly scar tissue, but lower lids show follicles; tissue taken from lower lids. Treatment irregular.

Case 6. K. A., female, 10 years old. Trachoma of 5 years duration. Mostly scar tissue; few follicles. Treatment as in Case 1.

Case 7. L. W., female, 9 years old. Mostly scar tissue, few small follicles. Duration of disease unknown. Treatment as in Case 1.

The eyes were washed with sterile saline, and the follicles were removed by Dr. Richards after novocain anesthesia (2 per cent). The medium used was the same as that employed for cultivation from the monkey conjunctiva, except that some plates were made with human blood. The plates, which had been brought from New York in a humidor and protected to some extent from drying by being strapped with adhesive tape, were again closed with adhesive after inoculation. They were transported to New York in the humidor and were 9 days old when examined.

Cultures of *Bacterium granulosis* were obtained from Cases 3 and 4, *i.e.*, from the two cases which had received no treatment. The human blood medium appeared to be as satisfactory as that made with horse blood. The cultures were typical of *Bacterium granulosis* morphologically and culturally and induced the characteristic conjunctival lesions in monkeys (Table 6). Subsequently their action on carbohydrates was determined and found to conform with that of the Albuquerque Strain 1 of Noguchi.

(*b*) *Santa Fé and Albuquerque Cases.*—A second expedition to the Indian Schools was made in September, 1929. We are indebted to the Indian Service for providing facilities for the preparation of culture medium at the U. S. Indian Hospital in Santa Fé. No advanced untreated cases of trachoma were available for cultural study either at Santa Fé or at Albuquerque, and experiments with treated cases proved negative. It is of interest in this connection that a recent report of the Indian Service (10) shows a marked decline in the incidence of trachoma among the New Mexico (Pueblo) Indians, owing apparently to the systematic treatment which is being carried out in the Pueblo villages.

TABLE 5

Monkey No.	Date	Culture inoc.		Incubation period	Result		Remarks
		Age	Temp.		Left	Right	
	1929	*days*		*days*			
P-1	June 15	57	4°-6°C. 44 days	73	+++	+++	Lesions persisting (8 months)
P-2	"	"	"	"	+++	+++	Died after 144 days
P-3	"	"	"	"	+++	+++	Died after 127 days
P-4	Sept. 28	162	4°-6°C. 149 days		–	–	
P-5	"	"	"		–	–	
P-6	"	"	"		–	–	
	1930						
P-7	Feb. 10	297	4°-6°C. 284 days		–	–	
P-8	"	"	"	8	++	++++	
P-9	"	75	Room 69 days		–	–	
P-10	"	"	"	8	+	+	

(*c*) *Leupp Cases.*—Through the kindness of Doctors Proctor and Richards, in cooperation with Commissioner Rhoads, of the Indian

TABLE 6

Inoculation of Bacterium granulosis from Fort Defiance Cases

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1929		*days*			
F. D. 1	June 10	Cultures I gen. 7 days semisolid II gen. blood agar 4 days, Case 3	77	+++	++++	Lesions progressed for 3 months. Discarded 5 months
F. D. 2	"	"		–	–	Reinoculated with pooled strains Cases 3 and 4 after 5 months. ++ lesions in 35 days
F. D. 3	"	"	64	+++	++++	Began to recede after 2 months. Discarded 7 months after inoc.
F. D. 4	"	"		–	–	
Chimpanzee "May"	"	"		–	–	Reinoculated with pooled strains Cases 3 and 4 after 5 months. Negative
F. D. 5	"	Similar cultures. Case 4	132	+++	+++	Lesions progressed for 2 months. Discarded 1 month later
F. D. 6	"	"	64	+++	++++	Lesions progressed for 3 months
F. D. 7	"	"	117	+	+	Transient lesions
F. D. 8	"	"		–	–	
F. D. 9	July 27	Case 4 cultures		–	–	Discarded after
		III, IV, V gen.		–	–	months
F. D. 10	"	7, 14, 19, 25		–	–	"
F. D. 11	"	days semisolid med. and blood agar		–	–	"
F. D. 12	Nov. 11	Pooled cultures. Cases 3 and 4 10 days semisolid	35	++	+++	
F. D. 13	"	"	35	+	+	Died 78 days after inoc.

TABLE 6—*Concluded*

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1929		*days*			
Passage from F. D. 3						
F. D. 14	Aug. 16	Conj. susp. No. F. D. 3		–	–	
F. D. 15	"	"		–	–	
F. D. 16	"	"		–	–	
F. D. 17	"	"		–	–	

Service, a search for untreated cases of trachoma was made in the Navajo territory in Arizona, where systematic treatment has been less successfully applied because of the nomadic habits of the Navajos. It was found that at the Indian School at Leupp the treatment had not been carried out for at least 6 months, no physician having been on duty regularly. Thirteen cases were selected by Dr. Richards for study. Of these, 6 were cases of granular conjunctivitis of recent onset in which the diagnosis was not altogether certain, but in which there had been no treatment whatever, 5 were unquestionably trachoma, and 2 showed evidence of the beginning scar tissue formation, indicating that they were in all probability trachoma. The clinical data are presented in Table 7.

The follicular material used for inoculation of the culture media was removed by Dr. Richards, the eyes being first washed with sterile saline and the lids anesthetized with 0.5 per cent novocain. The usual medium was employed, *i.e.*, blood agar plates containing horse or human blood and a mixture of carbohydrates. The latter ingredient was the mixture originally used by Noguchi (1). It had been sterilized fractionally for 10 minutes on 3 successive days at 103°C. in a pressure cooker. There was some sedimentation on cooling, and only the clear supernatant fluid was added to the medium. The plates, which were prepared in Santa Fé the day previous to their use in Leupp, were strapped with adhesive tape after inoculation and brought back to New York in a humidor. They were not opened until arrival in New York, 9 days after inoculation. The weather conditions on the day of inoculation were unfortunate, in that high winds prevailed, and much sand and dust blew in through the windows and skylight of the operating room, where the cultures were made, hence the plates were in most cases nearly overgrown with molds by the 9th day. A few, however, were less seriously contaminated, and colonies of *Bacterium granulosis* were found to be present. The follicle suspensions were brought back to New York and fresh plates inoculated.

TABLE 7

Case No.	Patient	Age	Diagnosis	Remarks	Culture
1	E. L.	7	Trachoma. Beginning scar tissue	Fairly recent case. Treated last year	–
2	M. N.	9	Doubtful (*i.e.*, no scar tissue)	May be beginning trachoma. Treated last year	–
3	P. T.	7	"	May be beginning trachoma. Treated last year	–
4	F. C.	14	Unquestionably trachoma. Scar tissue present	Old case. No treatment since last May	+
5	J. D.	10	"	Old case. Treated last year	+
6	H. W.	6	Recent case; some evidence of beginning scar tissue formation	Treated last year	+
7	M. C.	9	Recent case; some evidence of beginning scar tissue. Follicles in left eye only	Treated last year	–
8	B. H.	9	Unquestionably trachoma. Scar tissue present	No treatment since last May	–
9	H.	6?	Doubtful (*i.e.*, no scar tissue)	No treatment	+
10	I. M.	6?	"	" "	–
11	K. W.	7	"	" "	–
12	M. (Keems Canyon)	9	Unquestionably trachoma. Scar tissue present	" "	–
13	E. M.	10–11	Doubtful (*i.e.*, no scar tissue). Right eye only affected	" "	–

From the 4 cases which were regarded as unquestionably trachoma, *Bacterium granulosis* was obtained in 3. The organism was also obtained from Case 6, which showed evidence of early scar tissue formation, and from Case 9, which showed no scar tissue. The results of inoculation of the pooled cultures from Cases 5, 6, and 9 are recorded in Table 8. The lesions in Monkey L-6 are shown in Fig. 1.

4. Cultural and Biological Characteristics of the Various Strains

(*a*) *Viability.—Bacterium granulosis* remains viable for long periods on the semisolid ("leptospira") medium, and cultures 6 months old, kept at room temperature, with no other protection than the cotton plug, are readily transplantable. Even on a plain agar slant, on which the organism grows less readily, it has been found to remain transplantable for at least 49 days. It is probable that the bacterium lives as long as the medium contains a certain amount of moisture. Cultures kept in sealed ampoules for periods of 6 weeks to 10 months at 4° to 6°C., and for 69 days at room temperature, have proven viable and infective (Table 5).

TABLE 8

Inoculation of Bacterium granulosis from Leupp Cases

Monkey No.	Inoculated		Incubation period	Result		Remarks
	Date	Material		Left	Right	
	1929		*days*			
L-1	Nov. 22	Pooled cultures Cases 5, 6, and 9, I–II gen. semisolid med., blood agar plus carbohydrates, and hormone agar plus carbohydrates 48–72 hrs. old		–	–	
L-2	"	"	13	++	++++	++++ at 74 days
L-3	"	"	13	++	++	++ " " "
L-4	"	"		–	–	Died 13 days after inoc.
L-5	"	"	13	++++	++++	++++ at 74 days
L-6	"	"	13	++++	++++	++++ " " "

(*b*) *Cultural Characteristics.*—The cultural characteristics of the strains of *Bacterium granulosis* thus far isolated have been practically constant. In Tables 9 and 10 are shown the results of fermentation tests of the original strain and of the six strains isolated by us from Indian trachoma, as well as seven strains isolated by Finnoff and Thygeson (3) and one by Kendall (5), which these workers kindly sent us.

The tests recorded in Table 9 were made on Hiss serum water medium containing 1 per cent of the respective carbohydrates and litmus (about 0.01 per cent of a 25 per cent solution) as indicator, to which were added 10 per cent horse serum and 0.2 per cent horse hemoglobin; those shown in Table 10 were made on Dunham's peptone water containing 1 per cent carbohydrate and 1 per cent Andrade indicator (0.5 per cent acid fuchsin to which normal sodium hydroxide is added until the dye is decolorized). With the latter media a definite acid reaction is evident in 3 to 5 days, the lactose being the only one of the carbohydrates affected which shows no reaction until after this period. The use of Durham tubes shows that no gas is formed in any of the carbohydrate media.

Bacterium granulosis reduces nitrates to nitrites but forms no indole. It does not liquefy gelatin.

It has been found that after a few subcultures, all the strains of *Bacterium granulosis* grow well on nutrient agar (veal infusion agar containing 1 per cent of Witte's peptone and 0.5 per cent sodium chloride). The medium must be freshly prepared. Growth is more abundant if the agar is enriched by the addition of a carbohydrate mixture and horse blood or rabbit hemoglobin. A number of carbohydrates has been added individually to the horse blood agar medium in a concentration of 2 per cent; of these, media containing dextrose gave the best growth.

(*c*) *Reaction to Gram's Stain.*—Since *Bacterium granulosis* failed to retain an appreciable amount of the Gram stain (Sterling's gentian violet) when decolorized for 2 minutes and counterstained with dilute fuchsin, Noguchi regarded it as Gram-negative. It may be noted that Morax (11) regards it as Gram-positive, and since he apparently does not make use of a contrast stain in his technique, his point of view is comprehensible. However, after a contrast stain, even one as weak as Bismarck brown, no Gram stain can be detected in young, actively growing cultures. In old cultures, or cultures grown under unfavorable conditions (*e.g.*, in dextrose broth), numerous bizarre forms are seen which may retain the Gram stain. A form with an equatorial swelling, suggesting a central spore, is not uncommon in such cultures and usually stains Gram-positive, especially in the central portion.

(*d*) *Effect of Heat.—Bacterium granulosis* is rather sensitive to heating, being killed by exposure for 10 minutes to a temperature of 57°C. The thermal death point was tested by submerging sealed ampoules containing 0.5 cc. of a rich culture (5 days old on the semisolid medium) in water at temperatures varying from 52°C. to 65°C., and 5 and 10 minute exposures were made at each temperature. The whole amount of the heated culture was transferred, immediately after the heating, to fresh semisolid medium.

TABLE 9

Fermentation Tests. Hiss Serum Water Medium

	F. D. 3	F. D. 4	L. 5	L. 6	L. 9	Kendall
Dextrose	+	+	+	+	+	+
Levulose	+	+	+	+	+	+
Mannose	+	+	+	+	+	+
Saccharose	+	+	+	+	+	+
Raffinose	+	+	−	+	+	−
Inulin	+	+	−	−	−	+
Galactose	+	+	+	+	+	+
Maltose	+	+	+	+	+	+
Salicin	+	+	+	+	+	+
Xylose	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+
Dextrin	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+
Amygdalin	+	+	+	+	+	+
Lactose	+	+	+	+	+	±
Dulcitol	−	−	−	−	−	−
Rhamnose	−	−	+	+	+	+
Trehalose	−	−	+	−	−	+
Sorbitol	−	−	−	−	−	−
Inositol	−	−	−	−	−	−
None	−	−	−	−	−	−

+, color of indicator changed to red. No coagulation.

−, no change in color of indicator.

Readings recorded were made at 9 days. No change occurred after this time, and the tubes were discarded at 14 days.

F. D. = Fort Defiance. L. = Leupp.

TABLE 10

Fermentation Tests. Dunham's Peptone Water Medium

	Aq. 1	D. 1	D. 2	D. 4A	D. 5	D. 6	D. 7	F.D. 4	L. 4
Dextrose	++++	++++	+++	++++	++++	++++	++++	+++	+++
Levulose	+++	++++	+++	+++	++++	+++	++++	+++	+++
Mannose	+++	++++	+++	+++	++++	+++	++++	+++	+++
Saccharose	+++	++++	+++	+++	+++	++++	++++	++	+++
Raffinose	–	–	–	–	–	–	–	±	
Inulin	+	±	+	±	+	±	+	–	–
Galactose	+++	++++	++	++	++++	+++	++++	+++	++
Maltose	+++	+++	+++	+++	++++	+++	++++	+++	++
Salicin	++++	++++	++++	++++	++++	++++	++++	++++	++++
Xylose	++	++++	+++	+++	++++	+++	++++	++++	+++
Mannitol	++++	++++	+++	+++	++++	++++	++++	+++	+++
Dextrin	++++	++++	+++	+++	++++	++++	++++	+++	+++
Arabinose	++++	++++	+	+	++++	++++	++++	++++	+++
Amygdalin	+++	++	+	+	+++	++++	+++	+++	+++
Lactose	+	+	+	+	+	±	±	+	+
Dulcitol	–	–	–	–	–	–	–	–	–
Rhamnose	+++	++++	+	+	+++	++++	++++	++++	+++
Trehalose	++	+++	+	–	+++	+++	+++	+++	++
Sorbitol	–	–	–	–	–	–	–	–	–
Inositol	–	–	–	–	–	–	–	–	–

Aq. = Albuquerque.
D. = Strains isolated by Thygeson in Denver.
F. D. = Fort Defiance.
L. = Leupp.

(*e*) *Effect of Cocaine.*—The organism is also sensitive to the action of cocaine hydrochloride, being killed in 5 minutes by 4 and 5 per cent solutions, and in 15 minutes by weaker solutions (2 and 3 per cent). Similar concentrations of procaine hydrochloride (novocain) have no inhibitory effect on its growth. In obtaining material for cultivation, therefore, it is desirable to avoid long exposure to high concentrations of cocaine.

SUMMARY AND CONCLUSIONS

One of the strains of *Bacterium granulosis* isolated by Noguchi in 1926 has been maintained in culture and in monkeys and continues to be capable, after 3 years, of inducing a chronic granular conjunctivitis in monkeys. Cultures of this strain have been recovered from the monkey lesions as late as 3 years after inoculation and have been shown to reproduce the granular disease in monkeys.

Six additional strains of *Bacterium granulosis* have been isolated from cases of trachoma occurring in the Indian schools of Arizona. The cultures thus obtained are identical morphologically and culturally with those isolated by Noguchi and have induced the same chronic granular conjunctivitis in monkeys. Advanced untreated cases are more favorable for cultural study than treated cases.

Cultures of *Bacterium granulosis* kept on semisolid medium containing 10 per cent rabbit serum ("leptospira medium") remain viable for many months at room temperature, and sealed ampoules of such cultures have been found to retain their pathogenicity for the monkey conjunctiva for at least 69 days at room temperature and at least 284 days at 4° to 6°C.

Several additional cultural and biological characteristics of *Bacterium granulosis* have been described. Of outstanding importance is the fact that cocaine, in contradistinction to novocain, has a bactericidal effect on the organism. This fact, in view of the common use of cocaine for anesthesia, may explain the negative results of cultivation experiments reported by some workers.

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EXPLANATION OF PLATE 26

FIG. 1. *M. rhesus* L-6, 19 days after inoculation with pooled cultures from Leupp cases.

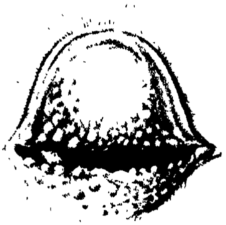
FIG. 2. *M. rhesus* 80, 2½ years after inoculation (1), showing presence of scar tissue.

FIG. 3. *M. rhesus* 5, 1½ years after inoculation, when the lid showed renewed activity of the lesions, and the left cornea became diffusely cloudy.

FIG. 4. *M. rhesus* 61, 57 days after inoculation with routine subcultures of Albuquerque Strain 1.

FIG. 5. *M. rhesus* 51, 65 days after inoculation with cultures recovered from *M. rhesus* 5.

Right Left



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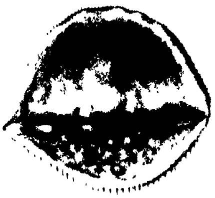

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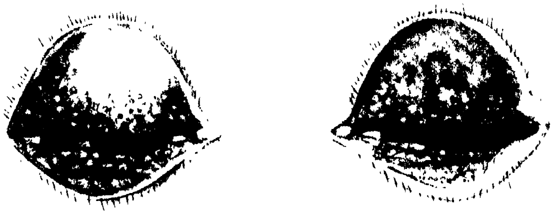

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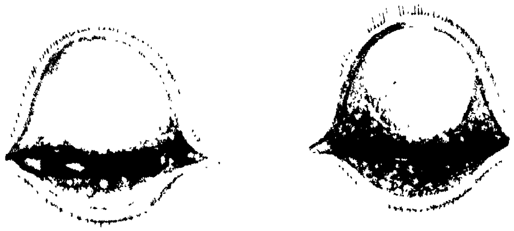

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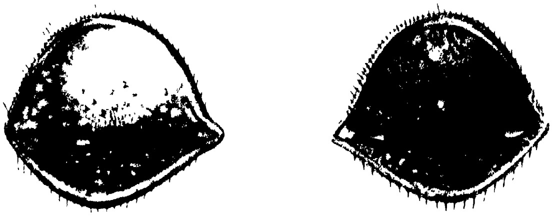

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(Tilden and Tyler *Bacterium granulosis* and trachoma)

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Vol. 52, No. 3, pp. 435–446]

THE KILLING OF CERTAIN BACTERIA BY X-RAYS

BY RALPH W. G. WYCKOFF, PH.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

PLATE 17

(Received for publication, May 24, 1930)

The experiments to be described are an extension to soft X-rays of those[1] previously made with cathode rays. They consist essentially of studies of the time rate of killing of single bacteria by the general radiation from a tungsten tube operated at low voltage and by the characteristic K-radiation of copper.

EXPERIMENTAL

The organisms *B. coli* and *B. aertryke* were chosen for these observations because they readily give the distribution of single cells that is needed if statistical counts are to have meaning. The bacteriological procedures were identical with those already described. As before, standard cultures were provided by Dr. L. T. Webster of this Institute. Spreads of single bacteria upon the surface of agar (ca 200 organisms per in.^{2}) were irradiated before multiplication could take place. After incubation, counts were made of the number of colonies growing out upon a stamped irradiated area and upon an equal and adjacent control area marked at the time of irradiation. From these data survival ratios have been calculated which, together with measurements of the X-ray intensity striking the agar plate, can be made to give information concerning details of the killing action of the rays. In order that the survival ratios obtained in this way should refer to enough organisms to be statistically significant, results from many plates have been averaged.

In the first group of experiments the X-rays used were the unfiltered general radiation from a Siemens Bucky tube having a tungsten target and a Lindemann glass window. The voltage across this tube was ca 12 KV peak and was provided by the unrectified output of a suitable transformer. A steady current of 8 MA was obtained by heating the cathode filament with an insulated storage battery. The bacteria were distant approximately 8 cm. from the tungsten target. Because of the long exposures required to give a sufficient killing action, only about 25 plates were irradiated in a day. Since the ratios of Table I and Text-fig. 1 are each averages of the counts upon 20 to 60 plates, several days were devoted to each experiment. These results obviously fall upon straight lines when plotted on semilogarithmic paper.

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[1] Wyckoff, R. W. G., and Rivers, T. M., *J. Exp. Med.*, 1930, 51, 921.

TABLE I

Survival Ratios with Bucky Tube

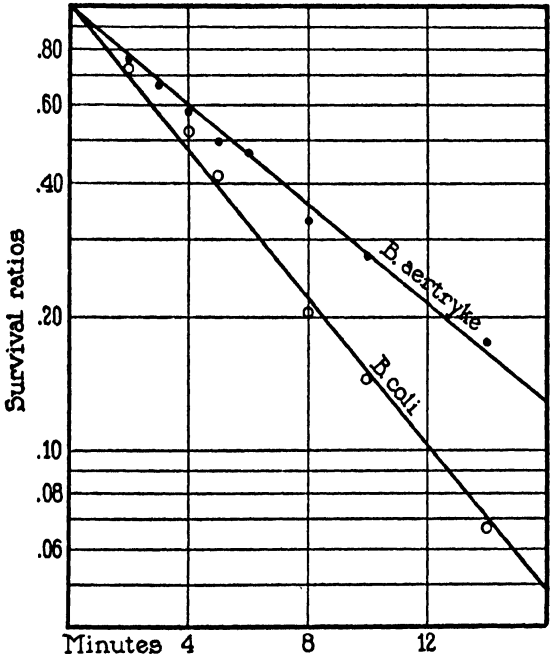
Time	Survival ratios	
	B. coli	*B. aertryke*
2 min.	0.750	0.768
3 "	—	.661
4 "	.526	.578
5 "	.416	.490
6 "	—	.466
8 "	.201	.329
10 "	.146	.274
12 "	.087	—
14 "	.064	.175

The survival ratios must therefore be expressed by an equation of the type

$$\frac{A_1}{A_0} = e^{-at}$$

Estimates of the intensity of the X-ray beam were made by measuring with a suitable chamber the ionization of air at the position of the irradiated bacteria. Subsequent experience has indicated that saturation currents were not obtained in these first experiments. No new determinations have been carried out because the general radiation of long wave length which constitutes the major output of a Bucky tube is poorly adapted to calculations of the mechanism by which cells are killed. It should be noted that the experiments of this group with *B. coli* and with *B. aertryke* were made under sufficiently different conditions so that no significance attaches to the relative killing rates found.

No commercially available electron-type X-ray tube supplies a monochromatic beam intense enough for experiments on the killing rates of bacteria. The self-rectifying gas tube developed in this laboratory and described[2] elsewhere is, however, suitable for this purpose and, equipped with a copper target, has been employed in a second series of experiments with *B. coli* and *B. aertryke.*



TEXT-FIG. 1. A plot of the survival ratios resulting from the use of soft genera radiation from a tungsten target X-ray tube (Table I).

In some instances the radiation from this tube has been filtered only by the 0.001 inch aluminum forming its window; in others a thickness of 0.0009 inch metallic nickel foil was interposed between the bacteria and the target. This filter cuts down the general radiation and eliminates 99 per cent of the K-β line at the same time passing half of the K-α doublet. The tube was operated at about 4 MA and 34 KV peak (unrectified wave). During a single series of experiments the variations in the current through the tube were less than 0.1 MA and its output of X-rays was found to be steady to within 1 per cent.

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[2] Wyckoff, R. W. G., and Lagsdin, J. B., *Radiology,* July, 1930, 15, 42.

Each series required the exposure of about 100 plates, the results of which were averaged to give the survival ratios of Tables II and III. As Text-figs. 2 and 3 indicate, these ratios fall upon straight lines when plotted on semilogarithmic paper. Such a result agrees with the experiments of Holweck[3] and Lacassagne[3] on *B. pyocyaneus* with silver L-radiation.

TABLE II

Survival Ratios with Unfiltered Copper Radiation

Time	Survival ratios			
	B. coli (1)	*B. coli* (2)	*B. coli* (3)	*B. aertryke*
5 sec.	—	0.749	—	—
10 "	—	.617	0.645	0.793
20 "	0.358	.329	.548	.583
30 "	.233	.206	.422	.387
40 "	.167	.154	.344	.349
50 "	.112	—	.265	.270
60 "	.100	.089	.193	.234
Ionization current/sec./cm.^{3}........	—	—	144.2 e.s.u.	—

TABLE III

Survival Ratios with Filtered Copper Radiation

Time	Survival ratios		
	B. coli (1)	*B. coli* (2)	*B. aertryke*
20 sec.	0.667	0.711	0.804
40 "	.367	.574	.523
60 "	.345	.362	.391
90 "	.182	.291	.211
120 "	.109	.212	.189
Ionization current/sec./cm.^{3}........	—	67.2 e.s.u.	67.2 e.s.u.

The experimental arrangement is shown in Fig. 1. During use the stamping device for the standard area A and the irradiated area B replaced the diaphragm

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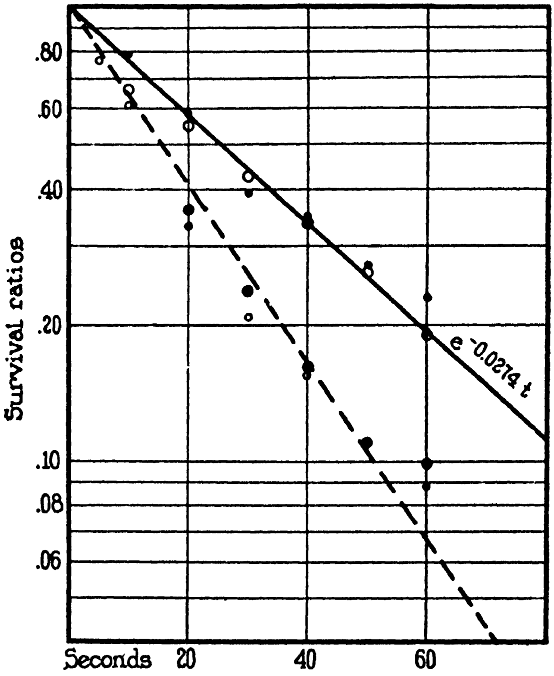
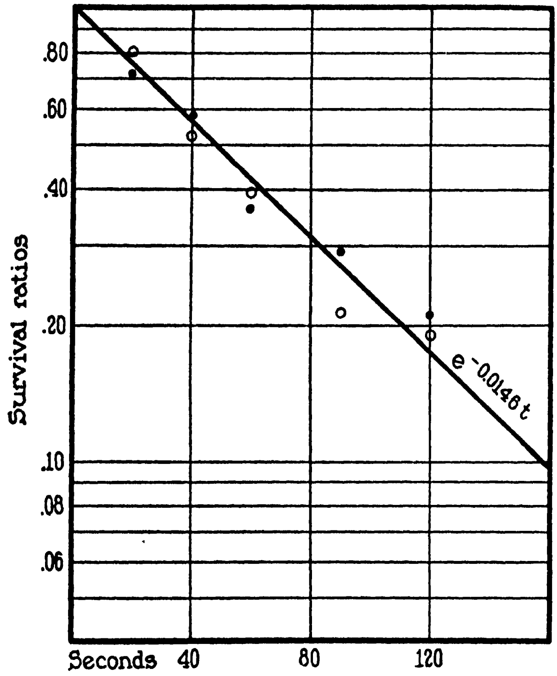
[3] Holweck, F., *Compt. rend.*, 1929, **188**, 197; Lacassagne, A., *Compt. rend.*, 1929, **188**, 200.

C under the X-ray tube D. When set up, the tube D was carefully centered with the aid of a fluorescent screen to insure that the X-ray beam covering B should be uniform. A Petri dish carrying the desired number of bacteria spread upon its agar surface was placed on the stand E and raised by the remote control rod F until the knife edges of A and B cut through the agar. On opening the metal shutter G, the surface marked by B could then be given an exposure of desired length. After lowering E, it and the dish were removed with the help of F. This remote control was needed because the X-ray tube itself operated continuously throughout the entire experiment. When in use, the tube was covered by a lead housing such as that shown at H and during exposures the door K was kept closed. These precautions are essential to the safety of the experimenter.



TEXT-FIG. 2. A plot of the data obtained by irradiating *B. coli* and *B. aertryke* with filtered copper rays. The small open, large black, large open and small black circles refer to experiments (1), (2), (3) and (4) of Table II.

The intensity of the X-rays striking the bacteria spread on the agar surface was obtained from measurements of the amount of ionization in air produced by these rays. Because of their absorbability, it was considered inaccurate to employ for this purpose a small enclosed type ionization chamber. Accordingly, an open air chamber of standard design but of small size was made. Its over-all length was 7.0 cm. The actual length of the amber-insulated collecting electrode was 1.31 cm., its effective length 1.39 cm. The grounded electrodes on either side were each 2.54 cm. long. All three electrodes were 3.8 cm. wide. The charged shield surrounding and facing these plates was 3.2 cm. distant. On account of the extremely great ionizing power of the X-rays used, saturation in a chamber of this type can be obtained only if beams of small cross-section are measured. The currents produced in this chamber were measured by the usual balance methods[4] employing



TEXT-FIG. 3. A plot of data obtained by irradiating *B. coli* and *B. aertryke* with the same dose of unfiltered copper rays. The small black and large open circles refer to experiments (2) and (3) of Table III.

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[4] See for instance, Makower, W., and Geiger, H., Practical Measurements in Radio-activity, London, 1912, 15.

a variable voltage and a standard condenser of known capacity. This capacity was obtained by comparison against a small standard condenser and checked by calculation from its accurately determined dimensions.

The intensity of the X-rays striking a small area of the agar surface was found by replacing the stamping device AB of Fig. 1 with a small lead stop, C, having an opening 0.117 cm. in diameter. The air chamber was brought as near to the stop as possible (ca 3 mm.) and the ionization produced by the X-ray beam passing through it was ascertained. Knowing the length of the measured column of ionized air and its absorption coefficient under the conditions of temperature and pressure of the experiment and for copper rays, it is obviously possible to calculate the approximate ionizing power of the beam at the irradiated surface. A typical calculation will make clear the procedure followed and the approximations introduced.

Area of stop in position of bacterium = 0.0107 cm.^2
Height of air column the ionization of which is measured = 1.39 cm.
Corrected density of air = 0.001165
Temperature = 26°C., Barometer = 747 mm. Hg.
Absorption coefficient μ/ρ of air for copper radiation = 8.43
Balancing voltage on potentiometer = 18.8 V in 20 sec.
1 e.s.u. potential = 0.33×10^{-2} volts
Measured capacity of standard condenser = 321.4 cm.

Therefore the observed ionization current is

$$i' = \frac{18.8 \times 0.33 \times 10^{-2} \times 321.4}{20 \text{ sec.}} = 0.997 \text{ e.s.u.}$$

Since $I/I_0 = e^{-\mu l}$ where l is the length in centimeters of the absorbing column and $\mu = 8.43 \times 0.001165$, a column of air 1 cm. long will absorb 0.721 as much as a similar column of length 1.39 cm. Hence the ionization produced per second in 1 cm. depth of air by the rays striking 1 cm.^2 of irradiated bacterial surface is taken as

$$i = \frac{i' \times 0.721}{0.0107} = 67.2 \text{ e.s.u. per cm.}^2 \text{ per sec.}$$

The intensities of the X-rays used in each of the three final standardized experiments when expressed in the foregoing units are recorded in Tables II and III.

Analysis

Existing knowledge of the properties of X-rays and of the mechanism of their absorption in inorganic matter makes it seem inevitable that their absorption in bacteria is a quantized process. Thus the X-rays incident upon a cell will either pass through without altering it or else they will give up one or more quanta whose energy content is connected with the wave length λ of the rays through the familiar relation

$$E = h\nu = h\frac{\lambda}{c}$$

where h is Planck's constant, ν is the frequency of the rays and c is the velocity of light. It is known that a high velocity electron is liberated as a result of such an absorption. This electron gives rise to a chain of ions in the matter through which it passes and to X-rays which, in their turn, liberate more ions of less and less energy. The volume within which this cluster of ions resulting from a single quantum absorption is freed increases rapidly with the magnitude of the original quantum but, in matter having the density of a bacterium, it is at greatest only a very small fraction of a cubic millimeter. The changes X-rays produce in protoplasm are naturally identified with the physico-chemical changes induced by this ionic shower.

The physical consequences of the absorption of an X-ray quantum so closely resemble those attending the direct absorption of a high velocity electron that it seems permissible to treat the killing action of X-rays by the same statistical analysis which has previously been used in studies of the killing of bacteria by cathode rays.

On this basis, the straight line obtained by plotting survival ratios upon semilogarithmic paper means that, on the average, the death of a bacterium is the result of the absorption of a single X-ray quantum. As the earlier analysis[5] has shown, survival ratios under these conditions can be calculated from the expression

$$\frac{A_1}{A_0} = e^{-\alpha t}$$

where t is the time and α is the expected, or average, number of quantum absorptions in unit time.

A more intimate picture of the killing process is to be had from a study of this expected number of hits. Under conditions of saturation, the air ionization chamber measures the total number of ions produced by the quanta absorbed in a known volume of air. If the number of pairs of ions liberated by a single quantum can be judged, then the number of quanta absorbed in this volume of air is known. Furthermore, if the ratio of the absorption coefficients of air and of a bacterium can be estimated, the *average* number of quanta absorbed by a single bacterium in unit time is readily computed from the size and shape of the organism. This has been done for experiments with filtered and unfiltered copper rays.

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[5] Wyckoff, R. W. G., and Rivers, T. M., *op. cit.*

The following typical calculations based upon the standardized experiment of Table III using *B. aertryke* will show how the average number of hits has been obtained and will make clear the approximations that have been introduced in the process.

$$67.2 \text{ e.s.u.} \approx 67.2 \times 0.21 \times 10^{10} \text{ ion pairs/sec.} = 1.411 \times 10^{11} \text{ ion pairs/sec./cm.}^3$$

A small amount of radiation is absorbed in the air between the diaphragm C and the collecting electrode of the air chamber. This absorption is readily calculated from the dimensions of the apparatus and the absorption coefficient ($\mu/\rho = 8.43$) of copper radiation. With the resulting correction it is found that 1.46×10^{11} ion pairs will be produced per second per cm.^{3} at the position of the irradiated bacteria. In the absence of direct experimental knowledge of the absorption coefficient of protoplasm, the mass coefficients of air and of living cells are customarily assumed equal. If this assumption, which cannot depart far from the truth, is made, the amount of absorption in 1 cm. thickness of air is about 27.9 times that of one bacterium. Both the *B. coli* and the *B. aertryke* have been taken as rods 0.5μ in diameter and 2μ long. Computation shows that to a first approximation one bacillus may be considered equivalent in absorption to a rectangular block of protoplasm 1×10^{-8} cm. in area, 0.42μ thick and of unit density. From these quantities the average number of ion pairs absorbed per bacterium per second becomes

$$\frac{1.46 \times 10^{11} \times 1 \times 10^{-8}}{27.9} = 52.3 \text{ ion pairs/sec./bacterium.}$$

In order to obtain the number of X-ray quanta absorbed per second it is necessary to know how many ions are liberated when one quantum is absorbed in air. The best available measurements[6] indicate that for X-rays of the quality used in these experiments about 35 volts are required to produce each electron pair. The voltage equivalent of the K-α lines of copper as computed from the familiar quantum relation

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[6] Kulenkampff, H., *Ann. d. Physik*, 1926, **79**, 97.

$$\text{Voltage (in KV)} = 12.34/\lambda(\text{in A}) = 12.34/1.537$$

is 8.029 KV. From this the number of ion pairs arising through the absorption of one quantum of Cu K-α radiation is

$$8029/35 = 229.$$

The average number, α, of quantum absorptions per second then is

$$52.3/229 = 0.228.$$

The observed survival ratios in Table III lead to a straight line having the equation $A_1/A_0 = e^{-0.0146t}$ (Text-fig. 2). It will be noted that this experimental $\alpha' = 0.0146$ is much smaller than the α calculated from ionization measurements.

DISCUSSION

Accepting the foregoing type of analysis as an essentially correct, if rough, description of what happens when X-rays strike bacteria, the smallness of the ratio α'/α when taken in connection with the linearly exponential character of the experimental results shows that though on the average the absorption of one quantum of these radiations is sufficient to kill a bacterium of either *B. coli* or *B. aertryke*, relatively few of the absorbed quanta are lethal. In the experiment just calculated 0.0146/0.228 or about one in 15.6 kills. In the standardized experiment of Table II using *B. coli* one in 17.8 is deadly ($\alpha = 0.489$, $\alpha' = 0.0274$). This agreement is satisfactory since it is scarcely to be expected that the results with filtered and unfiltered rays should be identical.

The fact that so many quanta can be absorbed by a bacterium without causing death apparently means that the vital elements within the cell which can be destroyed by a direct quantum hit are much smaller than the cell itself. If the volume through which the quantum acts were negligible compared to that of the vital element of the cell and if there were only one such element in a bacterium, then the "sensitive volume" that would have to be hit in order to bring about death would be α'/α. In the two standardized experiments with filtered rays this ratio is 0.064; in the series using unfiltered radiation it is 0.056. The spheres of action of these quanta cannot, however, be disregarded and the quantities α'/α are to an important degree measures of these regions of quantum action. Furthermore, if, as seems natural, the vital parts of the cell are identified with its chromatin material, more than one "sensitive volume" exists within it. It can nevertheless be concluded that the volume of the "vital elements" contained within one of the bacteria studied scarcely exceeds 0.05 of its entire volume. This estimate seems to be in serious conflict with the earlier conclusions of Holweck and Lacassagne with *B. pyocyaneus*. The published data are, however, too few to allow of a satisfactory comparison.

Within the limits of experimental error there is no difference between the sensitiveness of *B. aertryke* and *B. coli* to copper K-radiation (Text-fig. 2). A similar result was found when studying the killing action of cathode rays upon them.

Additional experiments are being carried out to ascertain in what way differences in X-ray wave length affect the killing of these organisms.

Valuable help in the carrying out of these experiments has been given by Charles G. Porskieves.

CONCLUSIONS

Both copper K X-rays and the soft general radiation from a tungsten tube operated at 12 KV kill *B. coli* and *B. aertryke* in a linearly exponential fashion. Within the experimental limits, the two organisms appear to be equally sensitive to these radiations.

By making use of the fact that X-ray energy is absorbed in quanta, an approximate picture can be formed of the mechanism of this destructive action. If the average numbers of quanta (α) absorbed per bacterium per second are calculated from measurements of air ionization using the quantities outlined in the text, survival ratios for these bacilli can be approximately represented by the equations

$$\frac{A_t}{A_0} = e^{-0.064\,\alpha\,t}$$

for filtered copper rays and

$$\frac{A_t}{A_0} = e^{-0.056\,\alpha\,t}$$

for unfiltered copper rays (peak voltage = 34 KV).

In terms of the foregoing interpretation this means that when death results, it is caused by the absorption of a single X-ray quantum of energy. Since only about one in twenty of the absorbed quanta kills, the sensitive cell constituents whose destruction leads to cell death must have a volume which is less than 0.06 of the bacterium itself.

EXPLANATION OF PLATE 17

Fig. 1. A photograph of the experimental arrangement for irradiating bacteria.

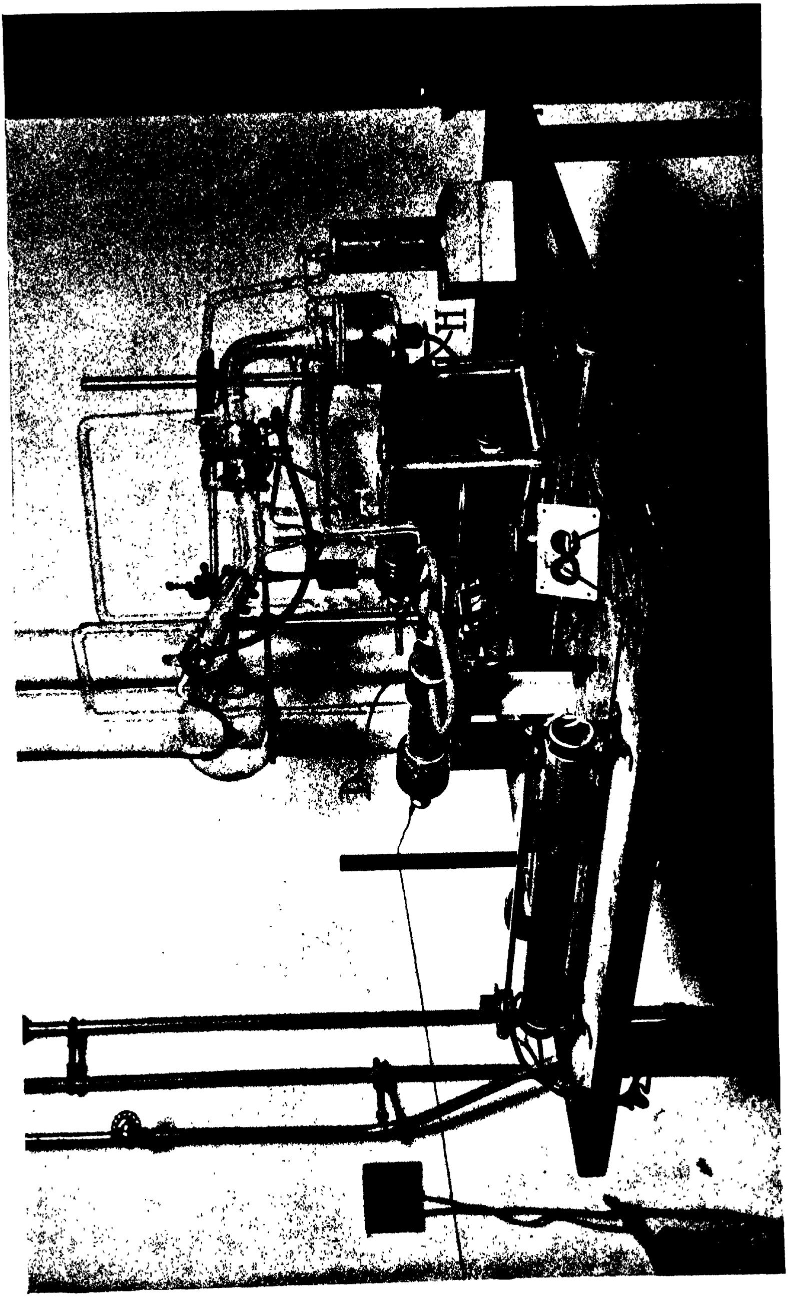

Fig. 1

(Wyckoff: Killing of certain bacteria by X-rays)

[Reprinted from THE PHYSICAL REVIEW, October 1, 1930, Vol. 36, No. 7, pp. 1116–1120]

THE ATOMIC SCATTERING POWERS OF NICKEL, COPPER AND IRON FOR VARIOUS WAVE-LENGTHS

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(Received for publication, June 26, 1930)

ABSTRACT

Atomic scattering powers are measured for the atoms in metallic nickel, copper and iron when they are reflecting the $K\alpha$ lines of molybdenum, copper, nickel and iron. These data show that the scattering power of an atom passes through a minimum at its K absorption limit and attains a maximum at, or near, its "resonance" wave-length. The atomic F-curves of the nickel atom in metallic nickel and in NiO are practically identical.

The data of this paper consist in measurements of the scattering powers of the metals nickel, copper and iron for the $K\alpha$ lines of molybdenum, copper, nickel and iron. Previous work from this laboratory with metallic copper and iron[1] showed that the reflecting powers of an atom might be considerably different for different wave-lengths. Studies[2] of NiO have indicated that the scattering power of nickel decreases as the wave-length used approaches its K critical absorption limit from the high frequency side and that it rises again after the limit is passed. The following results[3] suggest that this variation is generally to be expected.

F-curves for metallic nickel have been measured using the $K\alpha$ lines of molybdenum, copper, nickel and iron. The previous data from metallic copper and iron have also been extended through observations with copper, nickel and iron rays. The experimental procedures and the calculations leading to absolute F-values are the same[4] as those which have already been described. The $K\alpha$ lines of molybdenum were obtained by filtering the beam from a General Electric diffraction x-ray tube through a ZrO_2 filter; all other radiations were the unfiltered output of Siemens-Phoenix tubes equipped with appropriate targets. Calculation showed, however, that none of the measured $K\alpha$ reflections was, under the conditions of the experiment, contaminated by other wave-lengths.

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[1] A. H. Armstrong, Phys. Rev. **34**, 931 (1929).

[2] R. W. G. Wyckoff, Phys. Rev. **35**, 583 (1930).

[3] cf. R. W. G. Wyckoff, Phys. Rev. **35**, 215 (1930).

[4] A. H. Compton, X-rays and Electrons (New York, 1926), Chap. V; R. W. G. Wyckoff and A. H. Armstrong, Zeits. f. Krist. **72**, 319 (1929).

TABLE I

Relative Intensities of the Measured Powder Reflections from Metallic Nickel

Plane	Mo $K-\alpha$	Relative Intensities for		Fe $K-\alpha$
		Cu $K-\alpha$	Ni $K-\alpha$	
111	$374._6$	$431._2$	$412._4$	$226._5$
200	$181._6$	$192._1$	$185._7$	[100]
220	[100]	[100]	[100]	—
113	$102._9$	$107._4$	—	—
222	34.1	32.0	—	—
400	—	—	—	—
133	30.6	—	—	—
240	28.6	—	—	—
224	18.3	—	—	—
333 115	18.5	—	—	—
220 of NaCl	$571._7$	$107._7$	93.3	49.1

TABLE II

Radiation	Reflection	Intensity of reflection (220, NaCl = 100)	F for 110, Fe
Cu $K-\alpha$	110, Fe	51.9	11.48
Ni $K-\alpha$	110, Fe	37.5	9.77
Fe $K-\alpha$	110, Fe	$609._2$	13.45
Cu $K-\alpha$	220, Cu	94.2	—
Ni $K-\alpha$	220, Cu	88.4	—
Ni $K-\alpha$	200, Cu	$167._7$	—
Fe $K-\alpha$	200, Cu	$153._7$	—
Fe $K-\alpha$	111, Cu	$330._1$	—

The specimens studied were chemically pure, powdered metals which were passed through a fine meshed sieve and formed into suitable cakes by pressure applied in an ordinary vise. The surfaces of the preparations made in this way were filed and brushed before use in order to remove any superficial layer which might show a preferred orientation. Two samples each of nickel and copper, one of domestic and the other of foreign manufacture, gave concordant results. The iron used was an imported preparation of pure electrolytic metal.

The relative intensities of the various measured reflections are listed in Tables I and II together with the intensities of (220) of NaCl which has served as standard. Since the absolute reflecting power of no crystal is known for any of the wave-lengths used except Mo $K\alpha$, it has, as before, been assumed that F(220, NaCl) is independent of wave-length and equal[5] to 15.62. Though this assumption is almost certainly not strictly accurate, it probably does not depart far from the truth. When absolute F's shall have been determined for these longer wave-lengths, it will be easy to shift the present results to conform with them.

TABLE III

Absorption Coefficients Used in Calculating F-Values

Absorber	Density	Mo $K-\alpha$	*Absorbed Wave-length*		Fe $K-\alpha$
			Cu $K-\alpha$	Ni $K-\alpha$	
Nickel	8.90	423	432	549	825
Copper	8.95	—	468	586	886
Iron	7.92	—	2556	3124	570
NaCl	2.16	18.0_1	$161._2$	$196._2$	$306._8$

The F-values calculated from the intensity data of Tables I and II are dependent upon the absorption coefficients that are used. Experimental values exist for most of these coefficients. Except for metallic copper when absorbing Cu $K\alpha$ and metallic iron when absorbing Ni $K\alpha$ radiations, they are in satisfactory agreement with the values calculated by Jönsson's general formula.[6] Even in the most unfavorable case, the choice of extreme values of absorption coefficients makes a difference of only 0.4 of an absolute F-unit. For the sake of uniformity and completeness, calculated μ/ρ's as shown in Table III, have been employed throughout.

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[5] R. W. James and E. M. Firth, Proc. Roy. Soc. **117A,** 62 (1927).

[6] E. Jönsson, Uppsala Univers. Årsskrift (1928).

The resultant reflection F's are recorded in Tables II, IV and V and in Figure 1. Several comparisons are possible between these data and the results of previous measurements. The $F(220, \mathrm{Cu})$ for Cu $K\alpha$ is in fairly good agreement with existing information, but $F(110, \mathrm{Fe})$ for the same radiation falls considerably below the published[7] value.

TABLE IV

Absolute F-Values of the Nickel Atom in Metallic Nickel

Plane	sin θ/λ	Mo K-α (0.710 A)	Radiation		Fe K-α (1.932 A)
			Cu K-α (1.537 A)	Ni K-α (1.655 A)	
111	0.246	18.06	14.94	15.92	16.05
200	.284	16.89	13.70	14.71	14.69
220	.402	12.91	10.57	11.29	—
113	.471	11.10	8.73	—	—
222	.492	11.64	8.32	—	—
400	—	—	—	—	—
133	.619	8.37	—	—	—
240	.635	8.36	—	—	—
224	.696	7.48	—	—	—
333 115	.738	7.01	—	—	—

TABLE V

Absolute F-Values of the Copper Atom in Metallic Copper

Plane	sin θ/λ	Cu K-α (1.537 A)	Radiation Ni K-α (1.655 A)	Fe K-α (1.932 A)
111	0.241	16.60	—	14.59
200	.278	14.90	14.57	13.72
220	.392	11.53	11.18	—
113	.461	9.56	—	—
222	.481	9.10*	—	—

* The relative intensities of the reflections in this column (compared with 220) are those found by A. H. Armstrong (reference 1).

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[7] A. H. Armstrong, reference 1.

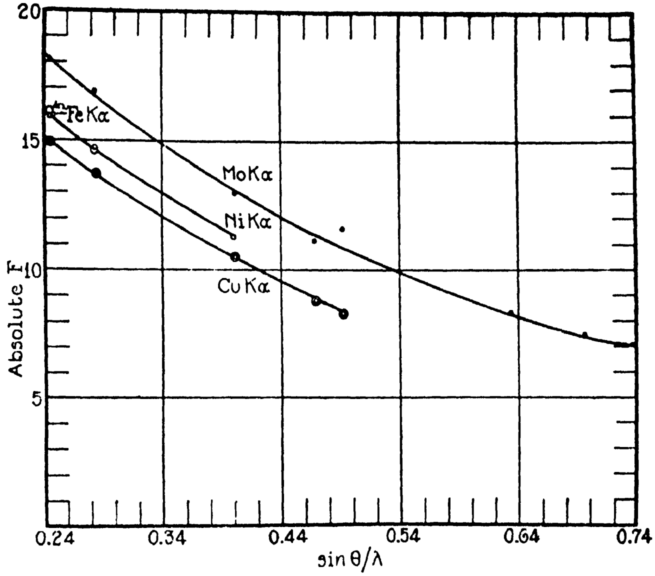

FIG. 1. The experimentally determined F-curves of metallic nickel for Mo $K\alpha$, Ni $K\alpha$ and Cu $K\alpha$ radiations. The two observed points for Fe $K\alpha$ rays are shown as large open circles.

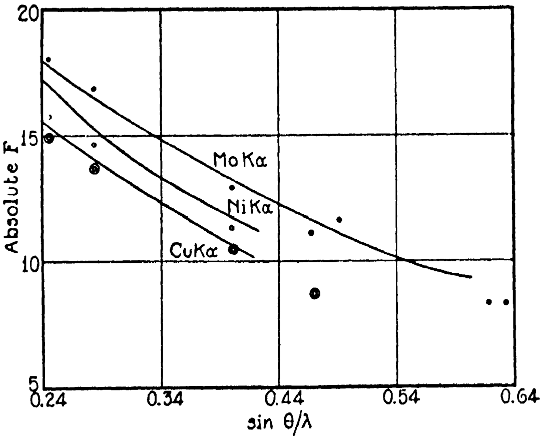

FIG. 2. The F-curves for the nickel atoms in NiO crystals are shown as full lines. Observed points for nickel atoms in metallic nickel appear as black circles (for Mo $K\alpha$), open circles (for Ni $K\alpha$) and ringed circles (for Cu $K\alpha$).

Additional observations with iron powder from the source which supplied the earlier experiments make it seem probable that there was some preferential orientation in the previous powder cake. Of interest is the comparison that can be made between the *F*-curves of metallic nickel and of the nickel ion[8] in NiO. This comparison is shown graphically in Figure 2 for Mo $K\alpha$, Cu $K\alpha$ and Ni $K\alpha$ rays. The agreement is especially close for the Mo $K\alpha$ and Cu $K\alpha$ curves.

Points on the *F*-curve for nickel with molybdenum radiation could be measured for reflections more complicated than (333; 115). Such reflections would be hard to determine with accuracy, however, because they are insignificant compared with the background of secondary nickel radiation excited by the Mo $K\alpha$ lines. The first, (111) reflection of nickel occurs at so great an angle ($\sin \theta/\lambda = 0.246$) that much of the complete *F*-curve of metallic nickel is of necessity extrapolated. This fact seems to prevent any important physical significance being attached to the electron-distribution curves that are calculated by Fourier analysis from the extrapolated forms of this *F*-curve.

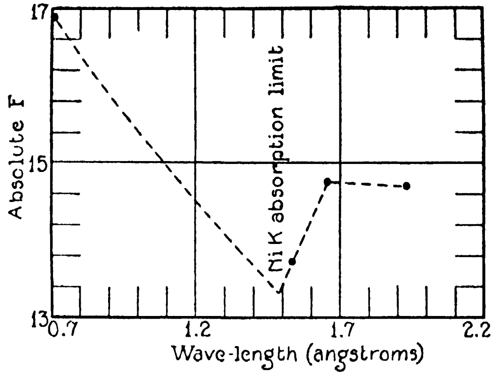

FIG. 3. The dotted curve indicates the variation of the scattering power of nickel with wave-length. The full circles are the measured values of the (200) reflection of metallic nickel.

The *F*-values of this paper show clearly the way in which the reflecting power of an atom varies with the wave-length of the x-rays scattered. The phenomena are illustrated by the (200) reflections of nickel as plotted in Figure 3. All of the other data of the present paper as well as those from NiO agree with this figure in showing that the scattering power of an atom is at a minimum close to its *K* critical absorption limit, that it attains a maximum at, or near, its "resonance" wave-length, but that the reflecting power falls off only slowly on the long wave-length side of this frequency. The photographs made by Mark and Szilard[9] of RbBr using strontium and bromine radiations are obviously an expression of the same variation.

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[8] R. W. G. Wyckoff, Phys. Rev. 35, 583 (1930).

It is hoped through additional experiments to put these scattering powers for long wave-lengths upon an accurate absolute scale.

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[9] H. Mark and L. Szilard, Zeits. f. Physik **33**, 688 (1925).

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ON WALDEN INVERSION

XIV. THE INFLUENCE OF SUBSTITUTING GROUPS ON OPTICAL ROTATION IN THE SERIES OF DISUBSTITUTED ACETIC ACIDS CONTAINING A PHENYL GROUP

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In a previous communication (1) we discussed the influence of substitution in a series of disubstituted acetic acid derivatives of the aliphatic series. In this series each substance contained only one group of significant polarity. In the present paper observations are presented on the derivatives of disubstituted acetic acids in which one of the substituting groups is aromatic, hence contains an additional polar group. The observations on this series of derivatives are of special importance as an aid for the interpretation of observations previously made on substitutions in secondary carbinols of mixed aliphatic-aromatic character. The substitution of the hydroxyl group by a halogen in the carbinols of that class is, under certain conditions, accompanied by Walden inversion. To be able to correlate configurations of the carbinols and halides is of fundamental importance for the purpose of singling out the cases in which a reaction of substitution is accompanied by Walden inversion.

In the case of the derivatives of dextro-disubstituted aliphatic acetic acid, the values of rotations descended with the changes in polarities of the substituting groups in the following order,

$$C{\equiv}N > COOC_2H_5 > COOH > CONH_2 > COCl > CH_2SO_3H > CH_2X >$$
$$CH_2SH > CH_2OH > CH_2NH_2$$

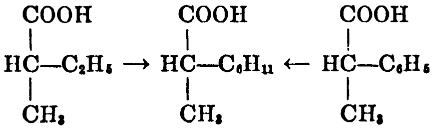
the direction of rotation being turned to the left for the carbinols and for the amines. It is self-evident that for the levorotatory series the changes of rotation should be of descending numerical value, becoming dextrorotatory for the carbinols and for the amines.

The events in the mixed aliphatic-aromatic series are markedly different. If the rotations of the first member of the series, namely dextro-phenylmethylacetic acid, and of the more significant derivatives of it are compared, the following descending order is observed.

$$-\mathrm{CO\overset{+}{O}H} > -\mathrm{CH_2\overset{+}{O}H} > -\mathrm{C\overset{-}{H_2}X} > -\mathrm{C{\equiv}N}$$

I.

Comparing these changes in rotations with those observed in the same derivatives of the dextro acids of the aliphatic series, one finds the order of change for $-\mathrm{C} \equiv \mathrm{N}$, CH_2X, CH_2OH, reversed. The question arises whether the two acids and their derivatives are configurationally of similar or of opposite series. With some degree of probability this question can be answered by hydrogenating dextro-phenylmethylacetic acid to the cyclohexylmethylacetic acid. This change brought about a significant drop in the value of rotation but did not alter the direction of rotation. On the assumption then that dextro-phenylmethylacetic acid has an analogous configuration to dextro-ethylmethylacetic acid, which might provisionally be given by the following figure



$$\begin{array}{ccccc} \mathrm{COOH} & & \mathrm{COOH} & & \mathrm{COOH} \\ | & & | & & | \\ \mathrm{HC{-}C_2H_5} & \rightarrow & \mathrm{HC{-}C_6H_{11}} & \leftarrow & \mathrm{HC{-}C_6H_5} \\ | & & | & & | \\ \mathrm{CH_3} & & \mathrm{CH_3} & & \mathrm{CH_3} \end{array}$$

II.

and as it is known that all the transformations given in Table I are accomplished without change in configuration, it is possible to follow the effect on rotation of the replacement of the higher aliphatic group by a phenyl group. The effect in the acid itself has already been mentioned. Another striking fact is the influence of the replacement in the nitrile. If the nitriles derived from the dextro-phenylmethylacetic acid are compared with those from the dextro-cyclohexylmethylacetic acids, it is noticed that the replacement of an aliphatic by a phenyl group brings about a change in the direction of rotation. Thus dextro-phenylmethylacetic acid leads to a levo-nitrile, whereas dextro-cyclohexylmethylacetic acid leads to a dextro-nitrile, as should be the case in the dextro-*n*-hexylmethylacetic acid series. The substances derived from the mixed nitriles all rotate in opposite directions from those of the aliphatic series, the only exception being the amine which, in the case of 2,2-phenylmethylethylamine, rotates in the same direction, as should be expected in the purely aliphatic substance.

The phenylethylacetic acid on hydrogenation to the cyclohexylethylacetic acid shows a drop in the value of the rotation, the direction remaining unaltered. Again in this case the replacement of the higher aliphatic radical by a phenyl radical in the nitrile leads to a change of the direction of rotation. This, however, is the only derivative of phenylethylacetic acid in which the direction of rotation is altered. On the other hand, if the numerical values of the rotations are compared, the same order of change is observed as in the methyl series; namely,

$$—COOH > —CH_2OH > —CH_2X > —C{\equiv}N$$

The most striking difference in the conduct of the derivatives of the two acids is noted in the reduction of the two nitriles to the corresponding amines. Both nitriles are levorotatory whereas the amines derived from them differ in their rotations; that derived from phenylmethylacetonitrile is levorotatory, the levorotation being enhanced, and that derived from the phenylethylamine is dextrorotatory. It would seem, then, that in the series of derivatives which are being described here the replacement of a methyl group by an ethyl group might change the direction of the rotation of the substance. This is rather an unexpected observation.

The dextro-phenylpropylacetic acid on hydrogenation leads to levo-cyclohexylpropylacetic acid (see Table II). This conduct is rather surprizing and somewhat puzzling. The fact that on hydrogenation the three dextro acids mentioned change their rotation in the same direction, namely towards the left, would warrant the conclusion that they are configurationally related in the sense given in figure (II), and if this assumption is correct, then it follows that levo-cyclohexylacetic acid is configurationally related to dextro-methyl- or ethylcyclohexylacetic acids. This result on the phenylpropylacetic acid again is unexpected and needs fur-

TABLE I

Molecular Rotations of Derivatives of Various Acids ($[M]_D^{25}$ *in Degrees*)

Series of:	$-\mathrm{C}(=\mathrm{O})\mathrm{OH}$	$-\mathrm{C}(=\mathrm{O})\mathrm{Cl}$	$-\mathrm{C}(=\mathrm{O})\mathrm{SH}$	$-\mathrm{C}(=\mathrm{O})\mathrm{OC_2H_5}$	$-\mathrm{C}(=\mathrm{O})\mathrm{NH_2}$	$-\mathrm{CN}$	$-\mathrm{CH_2NH_2}$	$-\mathrm{CH_2NH_2H}x$	$-\mathrm{CH_2Cl}$	$-\mathrm{CH_2OH}$	$-\mathrm{CH_2SH}$	$-\mathrm{CH_2SO_3H}$
Phenylmethylacetic acid.												
In ether..............	+89.5	+116.2	+156.4	+90.8		−16.6	−16.7		−0.5	+22.1	−14.1	
" absolute alcohol....	+82.0					−13.9	−27.6		−0.8	+12.7	−15.1	
" 75 per cent alcohol.	+81.4				+39.3							
" 50 " " " .	+81.2					−13.6						
Without solvent......	+96.8					−9.3	−23.9		$\frac{-2.5}{\delta}$			
In water.............								−35.4 (x = Br)				
Phenylethylacetic acid.												
In ether..............	+132.7	+163.0	+218.0	+139.3		−40.1	+14.5		+18.9	+45.2	+11.6	
" absolute alcohol....	+123.2					−38.1	−11.5		+16.8	+56.5	+10.5	
" 75 per cent alcohol.	+122.0				+32.4							
" 50 " " " .	+121.1											
Without solvent.......	+139.4					−29.0	+6.8		+17.8	+28.3		
In water.............								−4.9 (x = Br)				

Cyclohexylmethylacetic acid.												
In ether..............	−19.5	−13.3				−18.2						
" 75 per cent alcohol.	−17.5				−15.7	−20.3						
Without solvent.......						−19.6	Racemized.					
Phenylpropylacetic acid.												
In ether..............	+27.7	+40.4				−11.1						
" absolute alcohol....	+24.2											
" 75 per cent alcohol.	+23.9				+7.3		−5.2					
" 50 " " " .								−3.2 (x = Cl)				
Benzylphenylacetic acid.												
In ether..............	+82.8	+105.3		+155.7		+2.9	+57.6		+30.0	+39.4	+30.3	
" 75 per cent alcohol.	+97.7			+140.4	+92.8	−6.2	+57.1	+62.3 (x = Cl)	+28.4	+37.1		
" 50 " " " .												+29.3
Without solvent.......		+106.3		+162.1			$\frac{+53.8}{\delta}$					
In water.............												+30.8 (acid)
" "............												+39.7 (Ba salt)

ther substantiation. In the series of phenylpropyl carbinol the transformation could not be carried beyond the amine because of racemization. Attempts at the resolution of the amine were not successful.

The changes in the rotations of the derivatives of benzylphenylacetic acid could not be followed on substances obtained from one and the same starting material due to racemization which accompanied the transformation of the acid amide into the nitrile, the optical activity of the nitrile being so low that further transformation into optically active derivatives was no longer practicable. Fortunately, in this case it was possible to resolve the *d,l*-amine through its tartaric acid salt.

TABLE II

Molecular Rotations of the Derivatives of Various Acids ($[M]^{25}_{D}$)

Acid series.	Phenylacetic acids.	Corresponding cyclohexylacetic acids.
	degrees	*degrees*
Phenylmethylacetic..............................	−81.8	−19.5
Phenylethylacetic...............................	−130.8	−4.0
Phenylpropylacetic..............................	−29.0	+4.0

The rotation of the derivatives of the acid ending with the nitrile showed the same character of change as in the other two acids. The low dextrorotation of the nitrile in ether is not due to racemization entirely, as is seen from the fact that in 75 per cent of alcohol the rotation of the nitrile is reversed from that of the acid. The *d,l*-amine was resolved into its components by means of tartaric acid and the active forms were not correlated with the corresponding acetic acid. The rotations of the carbinol and of the chloride will be discussed below. Before proceeding with this discussion, however, the fact should be emphasized that in the series of derivatives of phenylmethylacetic acid and of its homologues, *the direction of rotation of substances of the same configuration differs from one homologue to another.* These changes can scarcely be accounted for by the differences in the polarities of the methyl, ethyl, or propyl groups.

The question of special interest to us, however, is that of the respective rotations of the carbinols and of the halides. In this respect a striking uniformity is observed in the three series in which the carbinols and the halides can be obtained in an optically active condition. *The changes in rotation on passing from the dextro-carbinol to the halide were towards the left.* In the case of 2,2-phenylmethylethanol and the corresponding halide, there was a reversion of the direction of rotation. In the other two cases the sign of rotation was not reversed, yet the drop in the rotation of the halide should be interpreted in the same sense and not on the basis of racemization for the reason that both substances are formed simultaneously by the action of nitrous acid and of nitrosyl chloride respectively. This change of rotation is analogous to that occurring in the derivatives of disubstituted acetic acids of the aliphatic series.

These observations lend further support to the conclusion reached by Levene and Mikeska regarding the configuration of methyl-, ethyl-, and propylphenyl carbinols. It was then assumed that the dextro-carbinol was configurationally related to the levo-halide. Indeed only on this assumption is the change of rotation on passing from the carbinol to the halide to the left, whereas the change is to the right when the rotations of the methyl- or ethylphenyl carbinols are compared, the latter being of a higher numerical value (2).

In the case of benzylphenyl carbinol the evidence furnished by the present observations is less decisive in view of the uncertainty as to the numerical value of the maximum rotation of the benzylphenylchloromethane.

CONCLUSIONS

1. There is a lack of uniformity in the direction of rotation of configurationally related homologous derivatives of substituted phenylacetic acids.
2. All dextrorotatory substituted 2-phenylethanols so far observed on passing to the corresponding halides show a change of rotation towards the left.
3. This observation supports the previous conclusion reached by us in regard to the correlation of the secondary aliphatic aromatic carbinols and of their corresponding halides.

EXPERIMENTAL

Resolution of d,l-Phenylmethylacetic Acid.—88 gm. of the acid were dissolved in 2700 cc. of hot acetone. 192 gm. of quinine were then added and the solution was allowed to stand overnight at room temperature. The quinine salt separated in crystalline form and amounted to 100 gm. 2 gm. of this salt were decomposed with hydrochloric acid and the rotation was determined on the acid thus obtained.

$$[\alpha]_D^{25} = \frac{-4.44^\circ \times 100}{1 \times 8.112} = -54.7^\circ \text{ (in ether).}$$

After repeated recrystallization from hot acetone, an acid was obtained which showed a rotation of

$$[\alpha]_D^{25} = \frac{-9.13^\circ \times 100}{1 \times 15.304} = -59.7^\circ. \quad [M]_D^{25} = -89.5^\circ \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-11.57^\circ \times 100}{1 \times 21.332} = -54.2^\circ. \quad [M]_D^{25} = -81.4^\circ \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = -59.1^\circ. \qquad [M]_D^{25} = -96.8^\circ \text{ (without solvent).}$$

On further recrystallization there was only a slight increase in rotation.

The rotatory power of the acid decreased with ionization. When the above acid was neutralized with 1 equivalent of sodium hydroxide, it showed a rotation of only

$$[\alpha]_D^{25} = \frac{-0.27^\circ \times 100}{1 \times 7.388} = -3.7^\circ. \quad [M]_D^{25} = -6.3^\circ \text{ (in water).}$$

Levo-Phenylmethylacetyl Chloride.—259 gm. of the corresponding acid, $[\alpha]_D^{25} = -59.7^\circ$ (in ether) were treated with 414 gm. of thionyl chloride (2 mols). The reaction mixture was allowed to stand overnight at room temperature and was then refluxed on the steam bath for 15 minutes. The unchanged thionyl chloride was removed under reduced pressure. The residue itself was not distilled inasmuch as previous experiments had shown that a slight racemization takes place even on distilling the product under highly reduced pressure. It had a rotation of

$$[\alpha]_D^{25} = \frac{-14.78^\circ \times 100}{1 \times 21.496} = -68.8^\circ. \quad [M]_D^{25} = -116.2^\circ \text{ (in ether).}$$

0.1017 gm. substance required 5.68 cc. 0.1 N $AgNO_3$ (titration).
C_9H_9OCl. Calculated. Cl 21.36. Found. Cl 19.82.

Levo-Phenylmethylacetamide.—50 gm. of the acid chloride described above, $[\alpha]_D^{25} = -68.8°$ (in ether), were dropped slowly with rapid stirring and thorough cooling into 150 cc. of concentrated alcoholic ammonia. The crude crystalline product was filtered off and the mother liquor concentrated to 30 cc. under reduced pressure. A second crop of crystals was obtained which was combined with the first. The amide was then recrystallized from hot water. Yield, 80 per cent. It melted at 92° and showed the following rotation.

$$[\alpha]_D^{25} = \frac{-2.97° \times 100}{1 \times 11.288} = -26.3°. \quad [M]_D^{25} = -39.2° \text{ (in 75 per cent alcohol).}$$

3.525 mg. substance: 9.410 mg. CO_2 and 2.245 mg. H_2O.
$C_9H_{11}ON$. Calculated. C 73.15, H 7.45.
Found. " 72.79, " 7.12.

Dextro-Phenylmethylacetonitrile.—50 gm. of the corresponding amide, $[\alpha]_D^{25} = -26.3°$ (in 75 per cent alcohol), were thoroughly mixed with 47 gm. (1 mol) of phosphorus pentoxide and distilled under a pressure of about 2 mm. The distillate was dissolved in ether, washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate. The ether was removed and the residue fractionated under a pressure of 8 mm. The nitrile distilled at 109°. Yield, 78 per cent.

$$[\alpha]_D^{25} = \frac{+2.23° \times 100}{1 \times 17.552} = +12.7°. \quad [M]_D^{25} = +16.6° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+1.83° \times 100}{1 \times 17.420} = +10.5°. \quad [M]_D^{25} = +13.7° \text{ (in 50 per cent alcohol).}$$

$$[\alpha]_D^{25} = +7.0°. \qquad [M]_D^{25} = +9.3° \text{ (without solvent).}$$

5.785 mg. substance: 17.440 mg. CO_2 and 3.425 mg. H_2O.
C_9H_9N. Calculated. C 82.44, H 6.87.
Found. " 82.21, " 6.62.

Dextro-2,2-Phenylmethylethylamine.—This was prepared by catalytic reduction of dextro-phenylmethylacetonitrile. Glacial acetic acid (300 cc.) dried over a few gm. of phosphorus pentoxide, was distilled directly into the absorption tube. 35 gm. of the nitrile, $[\alpha]_D^{25} = +5.61°$ (in ether), and 2 gm. of a platinum catalyst prepared according to the directions of Adams and Shriner were introduced into the tube by means of a dropping funnel. Care was taken to exclude all moisture.

The reduction was carried out under reduced pressure. After the nitrile and catalyst had been introduced into the absorption tube, before the shaking was begun, the air was withdrawn from the apparatus and replaced by hydrogen. The amount of hydrogen required for reduction of the nitrile to the corresponding primary amine was calculated to be 11,794 cc.

When 8700 cc. of hydrogen had been absorbed, the reaction became very slow and the catalyst was therefore reactivated by withdrawing the hydrogen from the apparatus, refilling it with oxygen, and shaking for 10 minutes. Each time when the reaction ceased the catalyst was again reactivated. After 11,500 cc. of hydrogen had been absorbed, the reaction almost ceased and could not be restimulated by further reactivation of the catalyst.

The solution was filtered and the filtrate concentrated under reduced pressure to about 30 cc. On diluting this solution with much water, a small amount of an oil separated which was extracted with ether. This proved to be unchanged nitrile. The mother liquor was rendered alkaline with sodium hydroxide and the amine was extracted with ether. The extract was washed with a little water and then dried over sodium sulfate. The ether was removed and the residue fractionated.

The first fraction distilled at 90° under a pressure of 12 mm. and the second at 185–186° under a pressure of 9 mm.

After redistillation, the first fraction (19.3 gm.) had the following rotation.

$$[\alpha]_D^{25} = \frac{+0.73^\circ \times 100}{1 \times 14.122} = +5.2^\circ. \quad [M]_D^{25} = +7.0^\circ \text{ (in ether).}$$

4.540 mg. substance: 13.320 mg. CO_2 and 3.925 mg. H_2O.
$C_9H_{13}N$. Calculated. C 80.00, H 9.63.
Found. " 80.00, " 9.67.

The second fraction proved to be a secondary amine. Total yield, 23.3 gm. (calculated 33 gm.).

In another experiment, 25 gm. of nitrile with $[\alpha]_D^{25} = +12.7^\circ$ (in ether) were reduced in 300 cc. of glacial acetic acid in the presence of 2 gm. of the same platinum catalyst. The first fraction showed a rotation of

$$[\alpha]_D^{25} = \frac{+1.44^\circ \times 100}{1 \times 11.592} = +12.4^\circ. \quad [M]_D^{25} = +16.8^\circ \text{ (in ether).}$$

$$[\alpha]_D^{25} = +17.7^\circ. \qquad [M]_D^{25} = +23.9^\circ \text{ (without solvent).}$$

The second fraction, a secondary amine, again amounted to about 20 per cent of the total yield.

Some of the primary amine was converted into the hydrobromide in dry ether. The precipitate was washed with ether and dried. It showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.78^\circ \times 100}{1 \times 4.752} = +16.4^\circ. \quad [M]_D^{25} = +35.4^\circ \text{ (in water).}$$

0.1000 gm. substance required 4.60 cc. 0.1 N $AgNO_3$ (titration).
0.1000 " " " 4.55 " 0.1 " HCl (Kjeldahl).
$C_9H_{14}NBr$. Calculated. N 6.48, Br 37.03.
Found. " 6.37, " 36.80.

Dextro-2,2-Phenylmethylethyl Chloride.—This was prepared by the action of nitrosyl chloride on dextro-2,2-phenylmethylethylamine. 15 gm. of the amine, $[\alpha]_D^{25} = +12.4^\circ$ (in ether), were dissolved in 100 cc. of dry ether, cooled to -55°, and then treated with an ethereal solution of nitrosyl chloride until the latter was no longer decolorized. Ice water was then added and the halide extracted with ether. The extract was washed with sodium hydroxide, then with water, and finally dried over sodium sulfate. The residue, obtained on the removal of the ether, weighed 7.6 gm. and showed a rotation of

$$[\alpha]_D^{25} = \frac{-6.32^\circ}{\delta} \text{ (without solvent).}$$

The crude material was submitted to fractional distillation. The first fraction distilled at 55–57° under a pressure of 0.8 mm. and weighed 4.7 gm. Optical rotation was as follows:

$$[\alpha]_D^{25} = \frac{-3.35^\circ}{\delta} \text{ (without solvent).}$$

$$[\alpha]_D^{25} = \frac{-1.12° \times 100}{1 \times 23.592} = -4.75° \text{ (in ether).}$$

The second fraction was not distilled, as previous experiments had shown that it could not be distilled without decomposition. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-1.42° \times 100}{1 \times 11.536} = -12.3° \text{ (in ether).}$$

To remove any carbinol that might have been formed during the reaction, the first fraction was dissolved in 20 cc. of dry pyridine and treated with 5 gm. of phthalic anhydride. The mixture was allowed to stand overnight at room temperature. The next day the solution was acidified with an excess of hydrochloric acid and extracted with ether. The ether extract was washed with dilute sodium hydroxide to remove the phthalic acid and the half ester of the same. The extract was then dried, the ether removed, and the residue distilled under a pressure of 1 mm. It boiled at 52–54°.

$$[\alpha]_D^{25} = \frac{+0.13° \times 100}{1 \times 38.944} = +0.33°. \quad [M]_D^{25} = +0.51° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+0.21° \times 100}{1 \times 40.960} = +0.51°. \quad [M]_D^{25} = +0.79° \text{ (in absolute alcohol).}$$

$$[\alpha]_D^{25} = \frac{+1.78°}{\delta}. \qquad [M]_D^{25} = \frac{+2.8°}{\delta} \text{ (without solvent).}$$

0.1152 gm. substance: 0.1036 gm. AgCl (Carius).

$C_9H_{11}Cl$. Calculated. Cl 23.22. Found. Cl 22.24.

Levo-2,2-Phenylmethylethanol.—The crude material obtained in the chlorination of dextro-2,2-phenylmethylethylamine, $[\alpha]_D^{25} = +12.4°$ (in ether), with nitrosyl chloride was treated with phthalic anhydride in pyridine solution, as described in the preceding experiment. The crystallized half ester of the carbinol was then isolated in the usual way. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-2.61° \times 100}{1 \times 15.800} = -16.5° \text{ (in ether).}$$

It was decomposed by boiling for 15 minutes with 3 mols of 30 per cent sodium hydroxide solution. An oil separated which was extracted with ether, washed with water, and then dried over sodium sulfate. The ether was removed and the oily residue distilled under reduced pressure. Only 0.3 gm. of the carbinol was obtained. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-1.79° \times 100}{1 \times 11.008} = -16.3°. \quad [M]_D^{25} = -22.1° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-0.76° \times 100}{1 \times 8.552} = -8.9°. \quad [M]_D^{25} = -12.1° \text{ (in absolute alcohol).}$$

4.325 mg. substance: 12.655 mg. CO_2 and 3.355 mg. H_2O.
$C_9H_{12}O$. Calculated. C 79.41, H 8.82.
Found. " 79.79, " 8.68.

Dextro-2,2-Phenylmethylethanthiol.—2 gm. of dextro-2,2-phenylmethylethyl chloride, $[\alpha]_D^{25} = +0.3°$ (in ether), were treated with 3 equivalents of a 10 per cent solution of alcoholic potassium hydrogen sulfide. The mixture was heated for 24 hours at 100° in a sealed tube. Much water was then added, the mercaptan was extracted with ether, washed with water, and dried over sodium sulfate. It was distilled under a pressure of 1.5 mm. The main part boiled at 70–71°, leaving a small residue in the flask.

The mercaptan showed the following rotation.

$$[\alpha]_D^{25} = \frac{+1.79° \times 100}{1 \times 19.320} = +9.3°. \quad [M]_D^{25} = +14.1° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+0.31° \times 100}{1 \times 3.128} = +9.9°. \quad [M]_D^{25} = +15.1° \text{ (in absolute alcohol).}$$

0.1216 gm. substance: 0.1761 gm. $BaSO_4$ (Carius).
$C_9H_{12}S$. Calculated. S 21.05. Found. S 19.89.

Dextro-Phenylmethylthiolacetic Acid.—14 gm. of the corresponding acid chloride, $[\alpha]_D^{25} = \frac{+11.69° \times 100}{1 \times 26.056} = +44.9°$ (in ether) were added drop by drop with stirring to 18 gm. of potassium hydrogen sulfide in 12 cc. of water. On heating the mixture on the steam bath a lively reaction took place. After all the chloride had been added, the reaction was heated on the steam bath for a few more minutes, then diluted with ice water and acidified. The oil which separated was extracted with ether, the extract washed and then dried over sodium sulfate.

On distillation the acid boiled at 136–138° under a pressure of 3 mm. Yield, 13.8 gm. It showed a rotation of

$$[\alpha]_D^{25} = \frac{+\ 10.03° \times 100}{1 \times 16.272} = +\ 61.6°. \quad [M]_D^{25} = +\ 102.3° \text{ (in ether).}$$

0.1362 gm. substance: 0.1862 gm. $BaSO_4$ (Carius).
$C_9H_{10}OS$. Calculated. S 19.27. Found. S 18.78.

Resolution of d,l-Phenylethylacetic Acid.—100 gm. of the acid were dissolved in 1000 cc. of hot 50 per cent alcohol. To this were added 154 gm. of cinchonidine and the solution was allowed to stand overnight at room temperature. The first fraction of the cinchonidine salt which separated in crystalline form weighed 121 gm. 2 gm. of this salt when decomposed with concentrated hydrochloric acid yielded an acid which showed a rotation of

$$[\alpha]_D^{25} = \frac{+\ 4.89° \times 100}{1 \times 16.460} = +\ 29.7° \text{ (in ether).}$$

The salt was recrystallized from 50 per cent alcohol until no further increase of the rotation of the free acid was obtained. It was then decomposed and the acid obtained was distilled. It showed a rotation of

$$[\alpha]_{D'}^{25} = \frac{+\ 3.58° \times 100}{1 \times 4.422} = +\ 81.0°. \quad [M]_D^{25} = +\ 132.9° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+\ 5.67° \times 100}{1 \times 7.624} = +\ 74.4°. \quad [M]_D^{25} = +\ 122.0° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = +\ 89.94°. \qquad [M]_D^{25} = +\ 139.4° \text{ (without solvent).}$$

The sodium salt had a rotation of

$$[\alpha]_D^{25} = \frac{+\ 0.86° \times 100}{1 \times 10.452} = +\ 8.2°. \quad [M]_D^{25} = +\ 15.3° \text{ (in water).}$$

Dextro-Phenylethylacetic Acid Ethyl Ester.—31 gm. of the corresponding acid, $[\alpha]_D^{25} = +80.9°$ (in ether), were poured slowly into a cooled mixture of 15 cc. of concentrated sulfuric acid with 100 cc. of absolute alcohol. The mixture was allowed to stand overnight at room temperature. The next day, ice and water were added. The precipitated oil was extracted with ether, and the extract washed with water, aqueous sodium hydroxide solution, and again with water. Then it was dried over sodium sulfate and the ether removed. The residue distilled at 80–85° under a pressure of 3 mm. It showed a rotation of

$$[\alpha]_D^{25} = \frac{+10.71^\circ \times 100}{14.844 \times 1} = +72.2^\circ. \quad [M]_D^{25} = +138.6^\circ \text{ (in ether).}$$

5.460 mg. substance: 14.895 mg. CO_2 and 3.905 mg. H_2O.
$C_{12}H_{16}O_2$. Calculated. C 74.96, H 8.38.
Found. " 74.39, " 8.00.

Dextro-Phenylethylacetyl Chloride.—32 gm. of the corresponding acid, $[\alpha]_D^{25} = +80.9^\circ$ (in ether), were treated with 100 gm. (4 mols) of thionyl chloride. A lively reaction took place without heating. The reaction mixture was allowed to stand overnight at room temperature and was then refluxed on the steam bath for 15 minutes. The unchanged thionyl chloride was distilled off under reduced pressure. The acid chloride itself was not distilled as a slight racemization was found to take place even on distilling the product under highly reduced pressure.

The product showed a rotation of:

$$[\alpha]_D^{25} = \frac{+13.89^\circ \times 100}{1 \times 15.596} = +89.0^\circ. \quad [M]_D^{25} = +162.5^\circ \text{ (in ether).}$$

0.1294 gm. substance: 6.90 cc. 0.1 N $AgNO_3$ (titration).
$C_{10}H_{11}OCl$. Calculated. Cl 19.67. Found. Cl 18.92.

Dextro-Phenylethylacetamide.—53.5 gm. of the corresponding chloride, $[\alpha]_D^{25} = +89.1^\circ$ (in ether), were added drop by drop with rapid stirring and thorough cooling to 200 cc. of concentrated alcoholic ammonia. The crude product was filtered off and the mother liquor concentrated under reduced pressure. A second crop of crystals was obtained which was combined with the first and the combined material was recrystallized from 50 per cent alcohol until free from ammonium chloride. Yield, 36 gm.

$$[\alpha]_D^{25} = \frac{+1.65° \times 100}{1 \times 8.308} = +19.9°. \quad [M]_D^{25} = +32.4° \text{ (in 75 per cent alcohol).}$$

0.0989 gm. substance: 0.2656 gm. CO_2 and 0.0710 gm. H_2O.
$C_{10}H_{13}ON$. Calculated. C 73.61, H 8.03.
Found. " 73.23, " 8.03.

Levo-Phenylethylacetonitrile.—32 gm. of the corresponding amide, $[\alpha]_D^{25} = +19.9°$ (in 75 per cent alcohol), were mixed with 28 gm. (1 mol) of phosphorus pentoxide and the mixture distilled under a pressure of 2 mm. The distillate was dissolved in ether, washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate. The ether was removed and the nitrile distilled under a pressure of 4.1 mm. It boiled at 56–57°. Yield, 20.6 gm.

$$[\alpha]_D^{25} = \frac{-2.56° \times 100}{1 \times 9.26} = -27.6°. \quad [M]_D^{25} = -40.1° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-3.33° \times 100}{1 \times 12.704} = -26.2°. \quad [M]_D^{25} = -38.1° \text{ (in absolute alcohol).}$$

$$[\alpha]_D^{25} = -20.0°. \quad [M]_D^{25} = -29.0° \text{ (without solvent).}$$

6.045 mg. substance: 18.235 mg. CO_2 and 3.770 mg. H_2O.
$C_{10}H_{11}N$. Calculated. C 82.76, H 7.58.
Found. " 82.26, " 6.97.

Dextro-2,2-Phenylethylethylamine.—This was prepared by catalytic reduction of levo-phenylethylacetonitrile. The reduction was carried out in exactly the same way as the reduction of phenylmethylacetonitrile.

6.5 gm. of the nitrile, $[\alpha]_D^{25} = -27.64°$ (in ether), were dissolved in 50 cc. of glacial acetic acid and 0.5 gm. of a platinum catalyst was added. In this case, the reduction proceeded to completion without reactivation. The amount of hydrogen absorbed was 2182 cc. (calculated 2007 cc.).

3.35 gm. of the primary amine were obtained. It distilled at 74° under a pressure of 2.6 mm. and showed the following rotations.

$$[\alpha]_D^{25} = \frac{+1.88° \times 100}{1 \times 19.306} = +9.7°. \quad [M]_D^{25} = +14.5° \text{ (in ether).}$$

$$[\alpha]^{25}_{D} = \frac{-1.16^\circ \times 100}{1 \times 15.024} = -7.7^\circ. \quad [M]^{25}_{D} = -11.5^\circ \text{ (in absolute alcohol).}$$

$$[\alpha]^{25}_{D} = +4.56^\circ. \qquad [M]^{25}_{D} = +6.8^\circ \text{ (without solvent).}$$

6.135 mg. substance: 18.035 mg. CO_2 and 5.515 mg. H_2O.

$C_{10}H_{15}N$.	Calculated.	C 80.53,	H 10.06.
	Found.	" 80.16,	" 10.06.

In this case, as in the reduction of the phenylmethylacetonitrile, about 20 per cent of the secondary amine was obtained.

Some of the above primary amine was converted to the hydrobromide in dry ether. The precipitate was washed with ether and dried. It showed a rotation of

$$[\alpha]^{25}_{D} = \frac{-0.06^\circ \times 100}{1 \times 2.824} = -2.1^\circ. \quad [M]^{25}_{D} = -4.9^\circ \text{ (in water).}$$

0.1000 gm. substance required 4.35 cc. $AgNO_3$ (titration).
0.1000 " " " 4.25 " 0.1 N HCl (Kjeldahl).

$C_{10}H_{16}NBr$.	Calculated.	N 6.08,	Br 34.78.
	Found.	" 5.95,	" 34.80.

Dextro-2,2-Phenylethylethyl Chloride.—This was prepared by the action of nitrosyl chloride on dextro-2,2-phenylethylethylamine. A solution of 10 gm. of the amine, $[\alpha]^{25}_{D} = +9.2^\circ$ (in ether), was dissolved in 100 cc. of dry ether, cooled to -55°, and then treated with an ethereal solution of nitrosyl chloride until the latter was no longer decolorized. Ice water was then added and the halide extracted with ether. The extract was washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate. The residue obtained on removal of the ether weighed 4.3 gm. and showed a rotation of

$$[\alpha]^{25}_{D} = \frac{+3.00^\circ \times 100}{1 \times 21.248} = +14.1^\circ \text{ (in ether).}$$

$$[\alpha]^{25}_{D} = \frac{+14.4^\circ}{\delta} \text{ (without solvent).}$$

The crude material was distilled under a pressure of 1 mm. The first fraction which boiled at 63–65° and of which 3.2 gm. were obtained, was dextrorotatory, as seen from the following figures.

$$[\alpha]_D^{25} = \frac{+\ 1.95° \times 100}{1 \times 15.848} = +\ 12.3° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+\ 13.08°}{\delta} \text{ (without solvent).}$$

The residue which could not be distilled without decomposition showed a rotation of

$$[\alpha]_D^{25} = \frac{+\ 4.49° \times 100}{1 \times 16.176} = +\ 27.8° \text{ (in ether)}$$

and analyzed as follows:

0.1214 gm. substance: 0.0471 gm. AgCl. Found. Cl 9.59.

To remove any carbinol that might have been formed during the reaction, the distillate was dissolved in 20 cc. of dry pyridine and treated with 3 gm. of phthalic anhydride. The mixture was allowed to stand overnight at room temperature. The next day the solution was acidified with an excess of hydrochloric acid and extracted with ether. The extract was washed with dilute sodium hydroxide in order to remove the phthalic acid and the half ester of the same. The extract was then dried, the ether removed, and the residue distilled under a pressure of 1 mm. It boiled at 62–64°.

$$[\alpha]_D^{25} = \frac{+\ 1.21° \times 100}{1 \times 10.840} = +\ 11.2°. \quad [M]_D^{25} = +\ 18.9° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+\ 0.85° \times 100}{1 \times 8.568} = +\ 9.9°. \quad [M]_D^{25} = +\ 16.8° \text{ (in absolute alcohol).}$$

$$[\alpha]_D^{25} = +\ 10.6°. \qquad [M]_D^{25} = +\ 17.8° \text{ (without solvent).}$$

0.1120 gm. substance: 0.0842 gm. AgCl (Carius).
$C_{10}H_{13}Cl$. Calculated. Cl 21.30. Found. Cl 18.59.

The analysis of the substance showed it to consist of 87.2 per cent halide and 12.8 per cent by-products. Inasmuch as its rotation is $[\alpha]_D^{2} = +11.2°$ (in ether), it follows that unless the by-product had a rotation of $[\alpha]_D^{25} = +87.2°$ (in ether), it could not account for the entire activity of the substance. Hence, it may be assumed that at least part of the optical activity was due to activity of the chloride.

In another experiment 3 gm. of amine with a rotation of $[\alpha]_D^{25} = +10.10°$ (in ether), which had been received from a previous chlorination experiment, were chlorinated and the resulting halide purified as described in the experiment above. The halide obtained showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.28° \times 100}{1 \times 5.056} = +5.53°. \quad [M]_D^{25} = +9.35° \text{ (in ether)}$$

and analyzed as follows:

0.1083 gm. substance: 0.804 gm. AgCl.
$C_{10}H_{13}Cl$. Calculated. Cl 21.30. Found. Cl 18.36.

The residue showed a rotation of

$$[\alpha]_D^{25} = \frac{+1.62° \times 100}{1 \times 14.072} = +11.5° \text{ (in ether).}$$

In this case, as in the previous one, the residue consisted of the halide with some other impurity as seen from the analysis given below.

0.1252 gm. substance: 0.0304 gm. AgCl. Found. Cl 6.00.

Dextro-2,2-Phenylethylethanol.—2 gm. of dextro-2,2-phenylethylethylamine,

$$[\alpha]_D^{25} = \frac{+1.88° \times 100}{1 \times 19.306} = +9.7° \text{ (in ether),}$$

were dissolved in 50 cc. of 95 per cent acetic acid and the solution was cooled in an ice-salt mixture. Nitrous oxide was then passed through the solution until the evolution of nitrogen ceased. Then 33 per cent sodium hydroxide was added until the solution was no longer acid to Congo red. Nitrous oxide was then again passed through the solution until the evolution of nitrogen almost ceased. Much water was then added and the precipitate was extracted with ether. The extract was washed with dilute sodium hydroxide, then with water, and finally dried over sodium sulfate. The ether was then removed and the residue distilled under reduced pressure. The alcohol was then purified as the ester of phthalic acid as described for the purification of other alcohols. The pure carbinol boiled at 95–96° under a pressure of 4 mm., and showed a rotation of

$$[\alpha]_D^{25} = \frac{+1.06° \times 100}{1 \times 3.536} = +30.0°. \quad [M]_D^{25} = +45.0° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+1.33° \times 100}{1 \times 3.536} = +37.6°. \quad [M]_D^{25} = +56.4° \text{ (in absolute alcohol).}$$

$$[\alpha]_D^{25} = +18.9°. \qquad [M]_D^{25} = +28.3° \text{ (without solvent).}$$

3.465 mg. substance: 10.195 mg. CO_2 and 2.875 mg. H_2O.
$C_{10}H_{14}O$. Calculated. C 80.00, H 9.33.
Found. " 80.23, " 9.28.

Dextro-2,2-Phenylethylethanthiol.—2 gm. of dextro-2,2-phenylethylethyl chloride, $[\alpha]_D^{25} = +11.2°$ (in ether), were treated with 3 equivalents of a 10 per cent solution of alcoholic potassium hydrogen sulfide. The mixture was heated for 7 hours at 100° in a sealed tube. Much water was then added and the mercaptan was extracted with ether, the extract washed with water, and then dried over anhydrous sodium sulfate.

On distillation, a fraction was obtained which boiled at 81° under a pressure of 1.3 mm. It showed the following rotation.

$$[\alpha]_D^{25} = \frac{+1.77° \times 100}{1 \times 25.240} = +7.0°. \quad [M]_D^{25} = +11.6° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+0.41° \times 100}{1 \times 6.456} = +6.4°. \quad [M]_D^{25} = +10.5° \text{ (in absolute alcohol).}$$

0.1130 gm. substance: 0.1276 gm. $BaSO_4$ (Carius).
$C_{10}H_{14}S$. Calculated. S 19.27. Found. S 15.51.

The second fraction distilled at 178–180° under a pressure of 1.5 mm. Optical rotation was as follows:

$$[\alpha]_D^{25} = \frac{-1.45° \times 100}{1 \times 4.664} = -31.1°. \quad [M]_D^{25} = -102.6° \text{ (in ether).}$$

This substance may be considered to be the disulfide, as shown from the following analysis.

0.1385 gm. substance: 0.2096 gm. $BaSO_4$ (Carius).
$C_{20}H_{24}S_2$. Calculated. S 19.39. Found. S 20.79.

2,2-Phenylethylethanesulfonic Acid.—About 2 gm. of dextro-2,2-phenylethylethyl chloride, $[\alpha]_D^{25} = +5.5°$ (in ether), were treated in a sealed tube with 2 gm. of potassium hydrogen sulfide in alcoholic solution. The mixture was heated for 6 hours at 100°. It was then isolated as described in the preceding experiment. Without distillation the substance showed a rotation of

$$[\alpha]_D^{25} = \frac{-0.61° \times 100}{1 \times 5.388} = -11.3° \text{ (in ether)}$$

and analyzed as follows:

0.1046 gm. substance: 0.1338 gm. $BaSO_4$ (Carius).
Mercaptan $C_{10}H_{14}S$. Calculated. S 19.27.
Disulfide $C_{20}H_{26}S_2$. " " 19.40. Found. S 18.53.

Presumably, the substance consisted of a mixture of the mercaptan and disulfide. By comparison with the previous experiment it may be assumed that this substance corresponds to a mercaptan with a rotation of

$$[\alpha]_D^{25} = +3.5°. \quad [M]_D^{25} = +5.8° \text{ (in ether).}$$

For the sake of conserving the material it was not distilled but was oxidized with barium permanganate in 95 per cent acetone. When the permanganate was no longer decolorized, the manganese dioxide was filtered off and the residue alternately washed with acetone and hot water. The combined filtrates were evaporated to dryness. The residue was washed with ether and then taken up with a little water. On addition of alcohol the salt precipitated as a semisolid mass which crystallized on stirring.

The substance analyzed as follows:

9.805 mg. substance: 1.931 mg. $BaSO_4$ (for Ba).
6.545 " " : 5.940 " " (" S).
Equimolecular mixture of acid and barium salt.
$C_{40}H_{54}O_{12}S_4Ba$. Calculated. S 12.91, Ba 13.85.
Found. " 12.44, " 11.73.

The analysis would seem to indicate that the substance was a molecular mixture of the free acid and the barium salt. Lack of material did not permit further purification.

To determine the rotation of the substance and of the corresponding free acid, we proceeded as follows: 0.1178 gm. of the salt was weighed in a flask and diluted to 2.5 cc. Rotation $[\alpha]_D^{25}$ in a 1 dm. tube was 1.30°. Hence the specific rotation of the substance was

$$[\alpha]_D^{25} = \frac{-1.30^\circ \times 100}{1 \times 4.712} = -27.6^\circ \text{ (in water).}$$

0.5 cc. of concentrated hydrochloric acid was then added to the solution directly in the polariscope tube. The total volume was then 3 cc. and the reading −1.11°. On the assumption that the original substance was an equimolecular mixture of acid and salt, it follows that the above corresponds to 0.1017 gm. of free acid per 3 cc.

Therefore,

$$[\alpha]_D^{25} = \frac{-1.11^\circ \times 100}{1 \times 3.390} = -32.7^\circ \text{ (in water).}$$

As neither the original mercaptan nor the oxidation product was obtained in pure form, the results cannot be regarded as reliable.

Levo-Phenylethylthiolacetic Acid.—7.8 gm. of the corresponding acid chloride, $[\alpha]_D^{25} = -23.7^\circ$ (in ether), were added drop by drop to a solution of 10 gm. of potassium hydrogen sulfide in 6 cc. of water. The solution was warmed on the steam bath whereupon a lively reaction took place. In a few minutes the reaction was completed. Ice was then added, the solution was acidified, and the acid extracted with ether, washed with water, and dried over sodium sulfate.

On distillation, a fraction was obtained which boiled at 124–125° under a pressure of 13 mm. Yield, 5.65 gm. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-\text{[illegible]}^\circ \times 100}{\text{[illegible]}\ 12\ 348} = -35.2^\circ. \quad [M]_D^{25} = -63.4^\circ \text{ (in ether).}$$

0.1554 gm. substance: 0.1523 gm. $BaSO_4$ (Carius).

$C_{10}H_{12}OS$. Calculated. S 17.77. Found. S 13.47.

Catalytic Reduction of Phenylmethylacetic Acid to Cyclohexylmethylacetic Acid.—2.92 gm. of .phenylmethylacetic acid with a rotation of $[\alpha]_D^{25} = +36.1^\circ$ (in ether), were dissolved in 100 cc. of glacial acetic acid. 0.3022 gm. of a platinum catalyst was then added. The reduction was carried out as previously described with an occasional reactivation of the catalyst.

When 1292 cc. of hydrogen were absorbed (calculated 1453 cc.) the reduction was discontinued. The solution was filtered and the filtrate concentrated under reduced pressure until an oily residue remained. This was taken up in ether, washed with water, and dried over sodium sulfate. The residue, after removal of the ether, was distilled under a pressure of 4.7 mm. The acid distilling at 120–121° showed a rotation of

$$[\alpha]_D^{25} = \frac{+0.80^\circ \times 100}{1 \times 10.228} = +7.8^\circ. \quad [M]_D^{25} = +12.2^\circ \text{ (in ether).}$$

On standing at room temperature the acid crystallized whereas the unreduced product remained liquid.

4.260 mg. substance: 10.795 mg. CO_2 and 3.930 mg. H_2O.
$C_9H_{16}O_2$. Calculated. C 69.23, H 10.25.
Found. " 69.10, " 10.32.

In another experiment, starting with an acid of $[\alpha]_D^{25} = -54.6^\circ$ (in ether), a reduced product was obtained showing the following rotation.

$$[\alpha]_D^{25} = \frac{-3.14^\circ \times 100}{1 \times 25.088} = -12.5^\circ. \quad [M]_D^{25} = -19.5^\circ \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{-2.24^\circ \times 100}{1 \times 19.928} = -11.2^\circ. \quad [M]_D^{25} = -17.5^\circ \text{ (in 75 per cent alcohol).}$$

The sodium salt, dissolved in water, showed the following rotation.

$$[\alpha]_D^{25} = \frac{-0.18^\circ \times 100}{1 \times 9.484} = -1.9^\circ. \quad [M]_D^{25} = -3.3^\circ.$$

Levo-Cyclohexylmethylacetyl Chloride.—142 gm. of the corresponding acid, $[\alpha]_D^{25} = -12.5^\circ$ (in ether), were mixed with 104 gm. of thionyl chloride (4 mols). A reaction, much livelier than that observed in the chlorination of the corresponding unreduced acid, took place without heating. The reaction mixture was allowed to stand overnight at room temperature and then refluxed on the steam bath for 15 minutes. The unchanged thionyl chloride was distilled off and the residue fractionated. It boiled at 54° under a pressure of 12 mm. Yield, 85 per cent. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-1.11° \times 100}{1 \times 14.624} = -7.6°. \quad [M]_D^{25} = -13.2° \text{ (in ether).}$$

0.1056 gm. substance required: 6.05 cc. 0.1 N $AgNO_3$ (titration).
$C_9H_{15}OCl$. Calculated. Cl 20.57. Found. Cl 20.33.

Levo-Cyclohexylmethylacetamide.—153 gm. of the corresponding acid chloride, $[\alpha]_D^{25} = -7.6°$ (in ether), were dropped slowly with rapid stirring and thorough cooling into 350 cc. of concentrated alcoholic ammonia. The crude product was filtered off and the mother liquor concentrated under reduced pressure. On further concentration of the mother liquor a second crop of crystals was obtained which was added to the first. The combined product was then recrystallized by dissolving it in a minimum amount of hot 99 per cent alcohol and precipitating it with water. Yield, 78 per cent. It was free from ammonium chloride and melted at 138–139°.

The optical rotation of the crude product was

$$[\alpha]_D^{25} = \frac{-0.55° \times 100}{1 \times 4.984} = -11.0°. \quad [M]_D^{25} = -17.1° \text{ (in 75 per cent alcohol).}$$

The optical rotation of the analyzed product was

$$[\alpha]_D^{25} = \frac{-0.45° \times 100}{1 \times 4.568} = -9.9°. \quad [M]_D^{25} = -15.3° \text{ (in 75 per cent alcohol).}$$

2.675 mg. substance: 6.695 mg. CO_2 and 2.575 mg. H_2O.
$C_9H_{17}ON$. Calculated. C 69.68, H 10.96.
Found. " 69.28, " 10.93.

Levo-Cyclohexylmethylacetonitrile.—50 gm. of the corresponding amide, $[\alpha]_D^{25} = -11.0°$ (in 75 per cent alcohol), were mixed with 45 gm. (1 mol) of phosphorus pentoxide and distilled under a pressure of 2 mm. The distillate was dissolved in ether, washed with dilute sodium hydroxide and then with water, and finally dried over sodium sulfate. The ether was removed and the nitrile distilled under a pressure of 3 mm. It boiled at 80°. Yield, 84 per cent. It showed a rotation of

$$[\alpha]_D^{25} = \frac{-2.72° \times 100}{1 \times 20.504} = -13.3°. \quad [M]_D^{25} = -18.1° \text{ (in ether)}.$$

$$[\alpha]_D^{25} = \frac{-2.53° \times 100}{1 \times 17.056} = -14.8°. \quad [M]_D^{25} = -20.3° \text{ (in 75 per cent alcohol)}.$$

$$[\alpha]_D^{25} = -14.3°. \qquad [M]_D^{25} = -19.7° \text{ (without solvent)}.$$

5.785 mg. substance: 17.440 mg. CO_2 and 3.425 mg. H_2O.
$C_9H_{15}N$. Calculated. C 78.82, H 10.94.
Found. " 78.70, " 11.49.

2,2-Cyclohexylmethylethylamine.—This was prepared by catalytic reduction of levo-cyclohexylmethylacetonitrile. The reduction was carried out exactly as in the case of the phenylmethylacetonitrile. 8.15 gm. of a nitrile, $[\alpha]_D^{25} = -14.3°$ (without solvent), were dissolved in 200 cc. of glacial acetic acid. 1.33 gm. of platinum catalyst were added. 2200 cc. of hydrogen were absorbed (calculated 2643 cc.).

The reaction mixture was filtered off from the catalyst and the glacial acetic acid removed under reduced pressure. The residue was diluted with water and extracted with ether to remove unchanged nitrile. The aqueous mother liquor was rendered alkaline with excess sodium hydroxide. It was extracted with ether, the ether washed, and then dried over sodium sulfate.

On distillation the amine boiled at 65–66° under a pressure of 2 mm. and was found to be optically inactive.

4.200 mg. substance: 11.775 mg. CO_2 and 5.110 mg. H_2O.
0.0352 gm. " : 6.53 cc. N (29°, 756 mm.).
$C_9H_{19}N$. Calculated. C 76.59, H 13.47, N 9.92.
Found. " 76.39, " 13.39, " 10.00.

Attempts have been made to resolve the inactive amine by means of tartaric-, camphorsulfonic-, and bromocamphorsulfonic acids, but without success.

Dextro-Cyclohexylethylacetic Acid.—This was prepared by catalytic reduction of phenylethylacetic acid. The procedure employed in the reduction of this acid was in every detail similar to the procedure used in the reduction of phenylmethylacetic acid.

3.0296 gm. of the acid, $[\alpha]_D^{25} = +79.8°$ (in ether), were dissolved *in 100 cc. of glacial acetic acid and* 0.2984 gm. of platinum catalyst was added. 1571 cc. of hydrogen were absorbed (calculated 1231 cc.). The acid was isolated as in the experiment mentioned above. The distilled product showed a rotation of

$$[\alpha]_D^{25} = \frac{+\,0.34° \times 100}{1 \times 14.372} = +\,2.4°. \quad [M]_D^{25} = +\,4.0° \text{ (in ether).}$$

3.345 mg. substance: 8.650 mg. CO_2 and 3.245 mg. H_2O.
$C_{10}H_{18}O_2$. Calculated. C 70.58, H 10.58.
Found. " 70.51, " 10.85.

Dextro-Cyclohexylpropylacetic Acid.—It was prepared by catalytic reduction of phenylpropylacetic acid. This experiment was also carried out according to the directions given for the reduction of the homologous phenylmethylacetic acid.

2.13 gm. of the acid, $[\alpha]_D^{25} = -16.3°$ (in ether), were dissolved in 100 cc. of glacial acetic acid and 0.2986 gm. of platinum catalyst was added. The total amount of hydrogen absorbed was 996 cc. (calculated 800 cc.). The distilled product showed a rotation of

$$[\alpha]_D^{25} = \frac{+\,0.22° \times 100}{1 \times 10.092} = +\,2.2°. \quad [M]_D^{25} = +\,4.0° \text{ (in ether).}$$

4.015 mg. substance: 10.425 mg. CO_2 and 3.895 mg. H_2O.
$C_{11}H_{20}O_2$. Calculated. C 71.73, H 10.86.
Found. " 70.80, " 10.85.

Resolution of d,l-Phenylpropylacetic Acid.—54 gm. of the acid were dissolved in a hot mixture of 300 cc. of 95 per cent alcohol and 8600 cc. of water. To this solution were then added 98 gm. of cinchonidine, whereupon the solution was allowed to stand for 36 hours at room temperature. The first fraction of the cinchonidine salt separated in crystalline form and amounted to 57 gm. 2 gm. of the salt were decomposed with concentrated hydrochloric acid. The free acid showed a rotation of

$$[\alpha]_D^{25} = \frac{+\,2.50° \times 100}{1 \times 16.784} = +\,14.9°. \quad [M]_D^{25} = +\,26.5° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+\,2.15° \times 100}{1 \times 16.088} = +\,13.4°. \quad [M]_D^{25} = +\,23.8° \text{ (in 75 per cent alcohol).}$$

From the mother liquor an acid was obtained which showed a rotation of

$$[\alpha]_D^{25} = \frac{-1.27^\circ \times 100}{1 \times 9.000} = -14.1^\circ. \quad [M]_D^{25} = -25.1^\circ \text{ (in ether).}$$

After two more recrystallizations of the first fraction, an acid was obtained with a rotation of $[\alpha]_D^{25} = +33.1^\circ$ (in ether). Further crystallization did not improve the rotation and the yield on this last fraction was only about 10 per cent.

Resolution of d,l-Benzylphenylacetic Acid.—The acid was resolved as a quinine salt by repeated recrystallization of the latter from 95 per cent alcohol. A fraction was finally obtained which yielded an acid with a rotation of

$$[\alpha]_D^{25} = \frac{-4.41^\circ \times 100}{1 \times 13.336} = -33.1^\circ. \quad [M]_D^{25} = -74.7^\circ \text{ (in ether).}$$

On decomposition of the mother liquors an acid was obtained with a rotation of

$$[\alpha]_D^{25} = \frac{+8.06^\circ \times 100}{1 \times 19.672} = +41.0^\circ. \quad [M]_D^{25} = +92.6^\circ \text{ (in ether).}$$

The above probably does not represent the absolute maximum rotatory power of the acid. For the sake of conserving the material no attempt was made to resolve it further.

The relation between the rotatory power of the acid in alcohol and ether, and of its sodium salt can be seen from the figures given below.

$$\text{For acid} \quad [\alpha]_D^{25} = \frac{-3.41^\circ \times 100}{1 \times 22.208} = -15.4^\circ. \quad [M]_D^{25} = -34.7^\circ \text{ (in ether).}$$

$$\text{For acid} \quad [\alpha]_D^{25} = \frac{-1.62^\circ \times 100}{1 \times 8.928} = -18.1^\circ. \quad [M]_D^{25} = -41.0^\circ \text{ (in 60 per cent alcohol).}$$

$$\text{For Na salt} \quad [\alpha]_D^{25} = \frac{-3.75^\circ \times 100}{1 \times 21.833} = -17.2^\circ. \quad [M]_D^{25} = -38.9^\circ \text{ (in water).}$$

The optical activity of the salt was determined in the presence of an excess of 1 mol of NaOH; that is, 1.9896 gm. of the above acid were treated with 2 mols of standard sodium hydroxide and the solution was diluted to 10 cc. The reading in a 1 dm. tub as $-3.75°$.

Dextro-Benzylphenylacetyl Chloride.—10 gm. of dextro-benzylphenylacetic acid,

$$[\alpha]_D^{25} = \frac{+6.94° \times 100}{1 \times 18.948} = +36.6°. \quad [M]_D^{25} = +82.8° \text{ (in ether)},$$

were treated with 20 gm. of thionyl chloride and allowed to stand at room temperature overnight. The next day the solution was refluxed on the steam bath for 15 minutes whereupon the excess of thionyl chloride was removed under reduced pressure. Without further purification the residue showed a rotation of

$$[\alpha]_D^{25} = \frac{+8.11° \times 100}{1 \times 18.864} = +43.0°. \quad [M]_D^{25} = +105.3° \text{ (in ether)}.$$

On distillation under a pressure as low as 0.2 mm. there was a slight racemization, so no attempt was made to purify the chloride further.

Dextro-Benzylphenylacetamide.—10 gm. of dextro-benzylphenylacetyl chloride, described above, ($[\alpha]_D^{25} = +43.0°$), were dropped slowly with thorough cooling and rapid stirring into about 5 mols of concentrated alcoholic ammonia. When all the chloride had been consumed, an equal volume of water was added. The amide precipitated immediately. It was then recrystallized from 50 per cent alcohol until free from ammonium chloride. When pure it melted at 134° and showed a rotation of

$$[\alpha]_D^{25} = \frac{+1.96° \times 100}{1 \times 4.752} = +41.2°. \quad [M]_D^{25} = +92.8° \text{ (in 95 per cent alcohol)}.$$

That some racemization had taken place during the reaction is evident from the fact that in another experiment an acid with a rotation of

$$[\alpha]_D^{25} = \frac{+9.32° \times 100}{1 \times 25.336} = +36.8°. \quad [M]_D^{25} = +83.1° \text{ (in ether)}$$

yielded an amide with a rotation of

$$[\alpha]_D^{25} = \frac{+3.96° \times 100}{1 \times 7.808} = +50.7°. \quad [M]_D^{25} = +114.1° \text{ (in 95 per cent alcohol)}.$$

0.1000 gm. substance: 4.01 cc. 0.1 N HCl (Kjeldahl).
$C_{15}H_{15}ON$. Calculated. N 6.22. Found. N 5.61.

Levo-Benzylphenylacetonitrile.—10 gm. of the dextro-benzylphenylacetamide described in the preceding experiment ($[\alpha]_D^{25} = +41.2°$) were mixed with 8 gm. of phosphorus pentoxide. The mixture was heated until molten. The nitrile was poured off, dissolved in ether, washed, and finally dried over anhydrous sodium sulfate. When the ether was removed the residue solidified. Without purification it showed an optical rotation of

$$[\alpha]_D^{25} = \frac{-0.38° \times 100}{1 \times 12.724} = -3.0°. \quad [M]_D^{25} = -6.8° \text{ (in absolute alcohol).}$$

The nitrile was then recrystallized several times from ether from which it crystallized well when cooled to about −55°. About 2 gm. of material were obtained which showed a rotation of

$$[\alpha]_D^{25} = \frac{-0.89° \times 100}{1 \times 11.316} = -7.9°. \quad [M]_D^{25} = -16.3° \text{ (in absolute alcohol).}$$

$$[\alpha]_D^{25} = \frac{+0.69° \times 100}{1 \times 18.616} = +3.7°. \quad [M]_D^{25} = +7.7° \text{ (in ether).}$$

0.1000 gm. substance: 4.60 cc. 0.1 N HCl (Kjeldahl).
$C_{15}H_{13}N$. Calculated. N 6.74. Found. N 6.44.

In the majority of cases the nitrile obtained by this method was either inactive or only slightly active.

Reduction of Benzylphenylacetonitrile.—15 gm. of the nitrile were dissolved in 300 cc. of 95 per cent alcohol. 30 gm. of sodium were then added with rapid stirring to the boiling solution. When all the sodium had dissolved, ice and water were added and then an excess of hydrochloric acid. The alcohol was removed under reduced pressure. An oil collected at the surface of the aqueous solution. This was extracted with ether and set aside for later examination. The aqueous mother liquor was made strongly alkaline with sodium hydroxide and the amine extracted with ether, dried over anhydrous sodium sulfate, and finally distilled under a pressure of 0.6 mm. It boiled constant at 128–129°. Yield, 5 gm.

The ethereal extract of the acid solution mentioned above was washed with water and dried over sodium sulfate. The residue crystallized on standing. On recrystallization from petrolic ether a snow-white substance was obtained which melted at 52°. This proved to be 2,3-diphenylpropane.

3.720 mg. substance: 12.550 mg. CO_2 and 2.685 mg. H_2O.
$C_{14}H_{14}$. Calculated. C 92.37, H 7.69.
Found. " 91.99, " 8.07.

Resolution of d,l-Benzylphenylethylamine.—23.5 gm. of the amine were poured into 16.7 gm. of tartaric acid dissolved in 500 cc. of water. The solution was allowed to stand at room temperature overnight. The crystalline precipitate was filtered off and recrystallized once more from 500 cc. of water. The salt was then decomposed with sodium hydroxide and the amine distilled under a pressure of 0.6 mm. The amine showed a rotation of

$$[\alpha]_D^{25} = \frac{+4.40° \times 100}{1 \times 16.264} = +27.1°. \quad [M]_D^{25} = +57.1° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = \frac{+5.26° \times 100}{1 \times 19.264} = +27.3°. \quad [M]_D^{25} = +57.6° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+31.21°}{\delta}. \qquad [M]_D^{25} = \frac{+65.85°}{\delta} \text{ (without solvent).}$$

In another experiment an amine was obtained with a rotation of

$$[\alpha]_D^{25} = \frac{+9.61° \times 100}{1 \times 23.960} = +40.1°. \quad [M]_D^{25} = +84.6° \text{ (in ether).}$$

Even this rotation could have probably been improved upon by further recrystallization but as in the previous experiments, no such attempt was made.

The levorotatory fraction was obtained from the mother liquors.

The hydrochloride of the amine was easily obtained by saturating an ethereal solution of the amine with dry hydrochloric acid gas. The rotation of the hydrochloride was

$$[\alpha]_D^{25} = \frac{-0.64° \times 100}{1 \times 3.996} = -16.0°. \quad [M]_D^{25} = -39.6° \text{ (in 75 per cent alcohol).}$$

$$[\alpha]_D^{25} = \frac{-0.62° \times 100}{2 \times 3.372} = -18.4°. \quad [M]_D^{25} = -45.6° \text{ (in water).}$$

0.1117 gm. substance: 4.43 cc. 0.1 N $AgNO_3$ (titration).
$C_{15}H_{18}NCl$. Calculated. Cl 14.34. Found. Cl 14.07.

Dextro-2,2-Benzylphenylethanol.—2 gm. of dextro-2,2-benzylphenylethylamine, $[\alpha]_D^{25} = +27.1°$, were dissolved in 60 cc. of 10 per cent sulfuric acid and cooled to 0°. Nitrous oxide was then passed through the solution until there was no further evolution of nitrogen. Much water was then added and the carbinol was extracted with ether. The extract was washed first with dilute sodium hydroxide, then with water, and finally dried over ether. The ether was removed and the residue distilled under reduced pressure. It was dextrorotatory as seen from the figures given below.

$$[\alpha]_D^{25} = \frac{+0.76° \times 100}{1 \times 4.088} = +18.6°. \quad [M]_D^{25} = +39.4° \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+0.68° \times 100}{1 \times 3.882} = +17.5°. \quad [M]_D^{25} = +37.1° \text{ (in 95 per cent alcohol).}$$

Dextro-2,2-Benzylphenylethyl Chloride.—This was prepared by the action of nitrosyl chloride on 2,2-benzylphenylethylamine. 15 gm. of the amine with a rotation of

$$[\alpha]_D^{25} = \frac{+5.26° \times 100}{1 \times 19.264} = +27.3°. \quad [M]_D^{25} = +57.6° \text{ (in ether)}$$

were dissolved in 100 cc. of dry ether. This was cooled to −55° and treated with an ethereal solution of nitrosyl chloride until the latter was no longer decolorized. Water was then added and the halide extracted with ether and finally dried over anhydrous sodium sulfate. When the ether was removed the residue weighed 7.5 gm. The unchanged amine was recovered and again treated with nitrosyl chloride. This was repeated until 13 gm. of the crude material were accumulated. To remove any carbinol that might have been formed during the reaction, the crude material was dissolved in 26 cc. of dry pyridine and treated with 9 gm. of finely pulverized phthalic anhydride. The mixture was allowed to stand at room temperature overnight. The next day the solution was acidified with excess of hydrochloric acid and extracted with ether. The ether extract was washed with dilute sodium hydroxide to remove the phthalic acid and the half ester of the same. The ether extract was then dried, the ether removed, and the residue distilled under a pressure of 0.3 mm. The halide distilled at 130–132°.

$$[\alpha]_D^{25} = \frac{+\ 0.89^\circ \times 100}{1 \times 7.308} = +\ 12.2^\circ. \quad [M]_D^{25} = +\ 28.1^\circ \text{ (in ether).}$$

$$[\alpha]_D^{25} = \frac{+\ 1.15^\circ \times 100}{1 \times 9.380} = +\ 12.3^\circ. \quad [M]_D^{25} = +\ 28.3^\circ \text{ (in absolute alcohol).}$$

$$[\alpha]_D^{25} = +\ 9.08^\circ. \qquad [M]_D^{25} = +\ 19.8^\circ \text{ (without solvent).}$$

0.1042 gm. substance: 0.0636 gm. AgCl (Carius).
$C_{15}H_{15}Cl$. Calculated. Cl 15.39. Found. Cl 15.10.

Levo-2,2-Benzylphenylethanthiol.—3 gm. of dextro-2,2-benzylphenylethyl chloride,

$$[\alpha]_D^{25} = \frac{+\ 1.19^\circ \times 100}{1 \times 9.160} = +\ 13.0^\circ. \quad [M]_D^{25} = +\ 30.0^\circ \text{ (in ether),}$$

were treated with 3 equivalents of a 10 per cent solution of alcoholic potassium hydrogen sulfide. The mixture was heated for 12 hours in a sealed tube at 100°. Water was then added and the mercaptan was extracted with ether, washed with water, and dried over anhydrous sodium sulfate. It was distilled under a pressure of 0.3 mm. It boiled at 123–127°.

$$[\alpha]_D^{25} = \frac{+\ 1.01^\circ \times 100}{1 \times 7.592} = +\ 13.3^\circ. \quad [M]_D^{25} = +\ 30.3^\circ \text{ (in ether).}$$

0.1406 gm. substance: 0.1090 gm. $BaSO_4$.
$C_{15}H_{16}S$. Calculated. S 14.03. Found. S 10.65.

Dextro-2,2-Benzylphenylethanesulfonic Acid.—1.5 gm. of the above 2,2-benzylphenylethanthiol were dissolved in 100 cc. of acetone and 2 cc. of water. This was then treated with an acetone solution of barium permanganate until it was no longer decolorized. The manganese dioxide was then filtered off, and washed alternately with hot water and acetone. The combined filtrates were evaporated to dryness under reduced pressure. The residue of barium salt was washed with ether to remove unchanged mercaptan. It was dissolved in water from which it separated on cooling as a viscous precipitate. This was then stirred several times with dry ether. It solidified on standing overnight. Without any further treatment the salt was pure as seen from the analysis given below. It showed the following rotation.

$$\text{For Ba salt } [\alpha]^{25}_{\mathrm{D}} = \frac{+0.54^\circ \times 100}{1 \times 4.680} = +11.5^\circ. \quad [M]^{25}_{\mathrm{D}} = +39.7^\circ \text{ (in water).}$$

6.970 mg. substance: 2.370 mg. $BaSO_4$ (for Ba).
0.0802 gm. " : 0.0546 gm. " (" S) (Carius).
$C_{30}H_{30}S_2O_6Ba$. Calculated. Ba 19.98, S 9.35.
Found. " 20.00, " 9.31.

To determine the rotation of the free sulfonic acid, 0.1170 gm. of the barium salt was treated with 0.5 cc. of concentrated hydrochloric acid and diluted to 3 cc. The reading in a 1 dm. tube was +0.35°. Hence,

$$[\alpha]^{25}_{\mathrm{D}} = \frac{+0.35^\circ \times 100}{1 \times 3.132} = +11.2^\circ. \quad [M]^{25}_{\mathrm{D}} = +30.8^\circ \text{ (in water).}$$

The rotation of the free acid was also determined in 50 per cent alcohol. 0.1356 gm. of the barium salt was treated with 0.5 cc. of concentrated hydrochloric acid and 2.5 cc. of absolute alcohol. The solution was then diluted to 5 cc. with water. In a 1 dm. tube the rotation was found to be +0.23°. Hence,

$$[\alpha]^{25}_{\mathrm{D}} = \frac{+0.23^\circ \times 100}{1 \times 2.178} = +10.6^\circ. \quad [M]^{25}_{\mathrm{D}} = +29.3^\circ \text{ (in 50 per cent alcohol).}$$

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ON THE RING STRUCTURE OF METHYLGLUCODESOSIDE

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The great instability of the ribodesosenucleosides as well as that of ordinary glucosides of 2-desoxy sugars made it necessary to investigate into the ring structure of these glucosides inasmuch as in regard to stability they resembled very much the γ-glucosides having the <1,4> structure. It is natural that the same question should also have interested Bergmann and while the present investigation was in progress there appeared a paper by Bergmann and Breuers dealing with this problem.[1] These authors prepared 4-glucosidoglucodesose from cellobiose. This disaccharide could not form a <1,4> glucoside and yet the methylglucoside prepared by them was characterized by a great instability. Thus, they attributed to the common methylglucodesoside the <1,5> ring structure. However, to make their proof really rigorous, they should have demonstrated that methylglucodesoside prepared from the disaccharide was identical with the glucoside prepared directly from the glucodesose. In view of this fact our work was brought to a conclusion and decisive evidence was furnished for the <1,5> structure of the methylglucodesoside. Our conclusion is based on the following facts.

Glucodesose was oxidized to the acid, which was converted into the lactone and the lactone was methylated. On the other hand, methylglucodesoside was exhaustively methylated. The methylated glucoside was converted into the corresponding sugar which was oxidized to the corresponding methylated sugar acid and this transformed into the corresponding lactone. In this way two trimethyl glucodesonic lactones were obtained, one starting from glucodesonic acid and the other from methylglucodesoside. To the first lactone, structure I may be attributed by analogy with other sugar acids.

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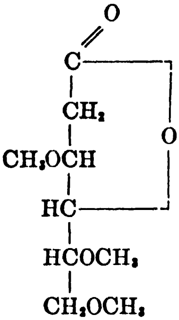
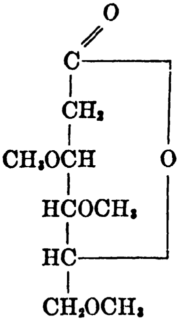
[1] Bergmann, M., and Breuers, W., *Ann. Chem.*, **470**, 51 (1929).



<1,4> lactone.

Crystalline.

I.



<1,5> lactone.

Liquid.

II.

The conclusion regarding the ring structure of the second lactone depends upon its identity or non-identity with the former. The first lactone crystallized readily. It was practically inactive in chloroform solution and had a rotation of $[\alpha]_D^{25} = +21.5°$ in benzene. The second lactone was a liquid and had a rotation of $[\alpha]_D^{25} = +87.5°$ in chloroform and of $[\alpha]_D^{29} = +88.2°$ in benzene. Hence the two lactones are not identical and it is warranted to assign the <1,5> ring structure to the second one and on the basis of this, also to the parent glucoside. *Thus the great instability of the 2-desoxyglucoside is due not to a <1,4> ring structure but to the reduced state of carbon atom 2.*

EXPERIMENTAL

2-Desoxy-d-Gluconic Acid.—The oxidation of 2-desoxy-*d*-glucose[2] was carried out following the method of Goebel.[3] The solutions used were prepared as follows: Solution A, 76 gm. of finely pulverized iodine were thoroughly mixed with 150 gm. of barium iodide,

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[2] Bergmann, M., Schotte, H., and Lechinsky, W., *Ber. chem. Ges.*, **55**, 165 (1922).

[3] Goebel, W. F., *J. Biol. Chem.*, **72**, 801 (1927).

1900 cc. of water were added, and the mixture was shaken until all the iodine and barium iodide were dissolved; Solution B, 124 gm. of $Ba(OH)_2 \cdot 8\ H_2O$ were dissolved in 1900 cc. of water.

The oxidation of the sugar was carried out as follows: 4 gm. of 2-desoxyglucose were dissolved in 322 cc. of Solution A. The mixture was cooled to $-5°$ and 487 cc. of Solution B were added from a dropping funnel in the course of 10 minutes with vigorous stirring. During the addition of the hydroxide the temperature was maintained at $-5°$. When all the barium hydroxide was added, the reaction was allowed to proceed for 5 more minutes. 22 gm. of lead carbonate were then added, and then 52 cc. of 25 per cent sulfuric acid. After a few minutes of stirring, enough lead carbonate was added to render the solution neutral to Congo red. The lead iodide was then filtered off and the filtrate concentrated to a small volume under reduced pressure. The last traces of hydroiodic acid were removed by treating the solution with a small excess of silver sulfate. Silver and lead were then removed with hydrogen sulfide. The solution was finally treated with an excess of barium carbonate and filtered. On concentration of the filtrate, the barium salt separated in crystalline form, and was filtered off. A second crop of salt was obtained from the filtrate by pouring it into a fairly large volume of methyl alcohol. The total yield of the Ba salt was 6.14 gm. It analyzed as follows:

4.485 mg. substance: 4.790 mg. CO_2 and 1.775 mg. H_2O.
6.470 " " : 3.055 " $BaSO_4$.
$C_{12}H_{22}O_{12}Ba$. Calculated. C 29.06, H 4.44, Ba 27.88.
Found. " 29.12, " 4.42, " 27.80.

In order to obtain the free acid, the salt was dissolved in water and the barium quantitatively removed with sulfuric acid. On concentration under reduced pressure the acid separated in crystalline form. It melted at 145° (uncorrected) and analyzed as follows:

5.705 mg. substance: 8.250 mg. CO_2 and 3.260 mg. H_2O.
$C_6H_{12}O_6$ Calculated. C 40.00, H 6.66.
Found. " 39.92, " 6.65.

The pure substance showed a rotation of

$$[\alpha]_D^{25} = \frac{+\ 0.15° \times 100}{1 \times 6.288} = +\ 2.4°\ \text{(in water)}.$$

1 hour later the rotation was

$$[\alpha]_D^{25} = \frac{+0.22^\circ \times 100}{1 \times 6.288} = +3.5^\circ.$$

3 days later it was

$$[\alpha]_D^{25} = \frac{+2.09^\circ \times 100}{1 \times 6.288} = +33.2^\circ.$$

At the end of that time equilibrium had not been reached.

Glucodesonic Lactone.—As was to be expected, it was found that the rate of lactone formation was accelerated by raising the temperature. It was therefore found expedient to make a 2 per cent solution of the acid and to heat it in sealed tubes for 24 hours in a steam-heated water bath at 100°. The solution was then concentrated to nearly dryness under reduced pressure and in order to remove as much water as possible, the residue was taken up in benzene and the latter removed by distillation under reduced pressure, the operation being repeated several times. On standing, beautiful colorless needles settled out on the walls of the flask. The contents of the flask were then dissolved in a large excess of acetone. Only a small part remained insoluble. As a rule, 2.0 gm. of the acid were used for each experiment. For the extraction with acetone several experiments were combined. The hot acetone extract was allowed to concentrate in a vacuum desiccator. The first crystals which formed on standing were composed in the main of the acid. From the mother liquor the lactone crystallized in heavy crystals, about four or five crops of crystals being obtained. For final purification it required five recrystallizations. Only then did the substance acquire a rather sharp melting point of 95–97° (uncorrected). The composition of the substance was the following.

4.475 mg. substance: 7.310 mg. CO_2 and 2.575 mg. H_2O.
$C_6H_{10}O_5$. Calculated. C 44.42, H 6.21.
Found. " 44.54, " 6.19.

The rotation of the substance was

$$[\alpha]_D^{25} = \frac{+1.36^\circ \times 100}{1 \times 2} = +68.0^\circ \text{ (in water).}$$

Trimethyl-d-glucodesonic Lactone <1,4>.—The above lactone was methylated once with methyl iodide and silver oxide first in acetonic solution and the product of this methylation remethylated directly in methyl iodide solution. 4.0 gm. of the crystalline lactone were dissolved in acetone and methylated with an excess of methyl iodide. The product of the second methylation was concentrated to a thick syrup under diminished pressure. The residue was taken up in very little ether and was allowed to stand in a desiccator at 10°. After several hours the substance turned into a crystalline mass. The substance was recrystallized from ether. It contracted at 59° and melted at 62° (uncorrected). It had the following composition.

4.710 mg. substance: 9.175 mg. CO_2 and 3.315 mg. H_2O.
4.280 " " : 14.75 " AgI (Zeisel).
$C_9H_{16}O_5$. Calculated. C 52.90, H 7.80, OCH_3 45.55.
Found. " 53.12, " 7.87, " 45.34.

There was no decisive rotation in chloroform.

The rotation was

$$[\alpha]_D^{25} = \frac{+0.43° \times 100}{1 \times 2} = +21.5° \text{ (in benzene).}$$

Trimethyl-d-Methyglucodesoside.—The methyl 2-desoxy-*d*-glucoside used in this experiment was prepared according to the directions of Bergmann and coworkers.[4] Without any purification other than the removal of the hydrochloric acid and the solvent, the glucoside was methylated with dimethyl sulfate and sodium hydroxide in the usual manner. For 10 gm. of the glucoside, 84 cc. of dimethyl sulfate and 84 cc. of 30 per cent sodium hydroxide were used.

The reaction flask was fitted with two graduated funnels, a return condenser, and a mechanical stirrer. 10 gm. of the glucoside were dissolved in 10 cc. of water and transferred into the reaction flask. 10 cc. of sodium hydroxide were then introduced through one of the dropping funnels and the mixture was heated to 50° in a water bath. The dimethyl sulfate and the sodium hydroxide were then allowed to flow at equal rate each from a separate funnel into the rapidly stirred solution. Care was taken to keep the solution slightly alkaline throughout the reaction. The water bath was maintained at 50° during the first half of the reaction, and at 60° during the latter half. When all the alkali and dimethyl sulfate had been added, the mixture was heated for another half hour to decompose all the dimethyl sulfate. The glucoside was then extracted with chloroform and the solution was washed free of alkali and dried over sodium sulfate. The chloroform was removed and the glucoside distilled under a pressure of 0.35 mm. It distilled at 86–90°. The rotatory power of the glucoside varied with the different preparations, ranging from $[\alpha]_D^{25} = +64°$ to $[\alpha]_D^{25} = +97°$ (in chloroform). The relative amount of α and β forms obviously varied with the various experiments. The pure methylated glucoside analyzed as follows:

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[4] Bergmann, M., Schotte, H., and Lechinsky, W., *Ber. chem. Ges.*, **55**, 169 (1922).

5.690 mg. substance: 11.180 mg. CO_2 and 4.570 mg. H_2O.
0.1053 gm. " : 0.4487 gm. AgI (Zeisel).
$C_{10}H_{20}O_5$. Calculated. C 54.54, H 9.09, OCH_3 56.31.
Found. " 54.34, " 9.01, " 56.24.

Trimethyl-d-Glucodesose.—10 gm. of trimethyl-2-desoxymethyl-*d*-glucoside were dissolved in 200 cc. of hot 0.1 N hydrochloric acid and heated for 10 minutes on the steam bath. The solution was cooled, treated with an excess of silver sulfate, and the filtrate freed from sulfuric acid and evaporated to dryness under reduced pressure. The residue was taken up in warm ether and filtered. The filtrate was transferred to a beaker and evaporated to dryness in a vacuum desiccator. The residue, obtained in a good yield, crystallized spontaneously. It was recrystallized from a mixture of ether and petrolic ether. When pure it melted at 58–61° and showed a rotation of

$$[\alpha]_D^{25} = \frac{+2.30° \times 100}{1 \times 3.864} = +59.5° \text{ (in water).}$$

$$[\alpha]_D^{25} = \frac{+1.28° \times 100}{1 \times 3.864} = +33.1° \text{ (at equilibrium in water).}$$

It analyzed as follows:

5.045 mg. substance: 9.705 mg. CO_2 and 3.935 mg. H_2O.
5.000 " " : 17.110 " AgI (Zeisel).
$C_9H_{18}O_5$. Calculated. C 52.42, H 8.73, OCH_3 45.14.
Found. " 52.45, " 8.72, " 45.17.

Trimethyl-d-Glucodesonic Acid.—The procedure for the oxidation of trimethyl-2-desoxy-*d*-glucose was similar to that used for the oxidation of 2-desoxy-*d*-glucose. Slight modifications were made, however: Solution B was added in the course of 5 minutes and the reaction was allowed to proceed for 5 minutes more before the lead carbonate and sulfuric acid were added. The proportions of the various reagents used were exactly the same. The salt, which was obtained as a viscous residue after evaporation to dryness, was taken up with a little dry methyl alcohol and filtered. The filtrate was concentrated and then poured into a mixture of methyl alcohol and ether. The Ba salt separated as a white amorphous powder. It was pure without further treatment as indicated by the analysis:

7.170 mg. substance: 9.680 mg. CO_2 and 3.650 mg. H_2O.
11.030 " " : 4.530 " $BaSO_4$.
$(C_9H_{17}O_6)_2Ba$. Calculated. C 37.26, H 5.90, Ba 23.70.
Found. " 36.81, " 5.69, " 24.16.

This salt showed the following rotation.

$$[\alpha]_D^{25} = \frac{+ 1.45^\circ \times 100}{2 \times 4.000} = + 18.1^\circ \text{ (in water).}$$

The acid showed the following rotations.

0.0914 gm. of salt was dissolved in 1 equivalent of 0.1 N hydrochloric acid and the solution was diluted to 5 cc. with water.

$$[\alpha]_D^{25} = \frac{+ 0.62^\circ \times 100}{2 \times 1.4014} = + 22.1^\circ.$$

0.0756 gm. of salt was dissolved in 1 cc. of 36 per cent hydrochloric acid (excess) and the solution was diluted to 2.5 cc. with water.

$$[\alpha]_D^{25} = \frac{+ 1.34^\circ \times 100}{1 \times 2.3184} = + 57.8^\circ.$$

Trimethyl-d-Glucodesonic Lactone <1,5>.—The above barium salt was dissolved in water and the barium removed quantitatively. The solution was then concentrated under reduced pressure. The residue did not dissolve completely in ether; hence it was taken up in acetone and concentrated at 0.02 mm. pressure, the temperature of the bath being 80°. This residue analyzed fairly well for the lactone (C = 52.81 and H = 7.48). The substance was then taken up in ether and the solution filtered from a slight insoluble precipitate. The ether was then removed by distillation and the residue distilled. It boiled at 137° under a pressure of 0.02 mm. This boiling point, however, is not regarded as definitely correct in view of the small quantity of material available. The distillate analyzed correctly for the lactone.

4.870 mg. substance: 9.470 mg. CO_2 and 3.500 mg. H_2O.

$C_9H_{16}O_5$.	Calculated.	C 52.90, H 7.80.
	Found.	" 53.02, " 8.04.

The rotation of the substance was as follows:

$$[\alpha]_D^{25} = \frac{+3.5^\circ \times 100}{1 \times 4} = +87.5^\circ \text{ (in chloroform).}$$

$$[\alpha]_D^{29} = \frac{+0.90^\circ \times 100}{1 \times 1.02} = +88.2^\circ \text{ (in benzene).}$$

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THE ACTION OF BENZOIC PERACID ON SUBSTITUTED GLUCALS

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The observations to be reported in this communication are the outcome of experiments performed with the aim of converting certain glucose derivatives into the corresponding derivatives of mannose.[1] A convenient method for such transformation was given by Bergmann and Schotte who converted glucal into mannose[2] by the action of benzoic peracid. The reaction proceeded in the same sense in the case of glucals substituted in position (4), 4-glucosidomannose being obtained from cellobial[3] and 4-galactosidomannose[4] from lactal.

However, we now find that when 3-methylglucal and triacetylglucal are acted upon by benzoic peracid the reaction proceeds abnormally and yields glucose derivatives. As the glucal is substituted on carbon atom (3) in both of these cases, it seems that the addition of the hydroxyls to the carbon atoms of the glucal is directed by the character of the groups attached to carbon atom (3).[5] Although this abnormal reaction was disappointing for our present purpose, it may be of service on other occasions.

In the case of the triacetylglucal, moreover, the benzoic peracid added directly to the double bond and yielded a benzoyl triacetylhexose. The same product was obtained in about equal yield when the reaction was performed in the presence of water. At the moment we are concerned only with the fact that the substance is a derivative of glucose, but we intend to report later on its structure.

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[1] In this connection we may mention that we have obtained from acetobromomannose (from the β-pentacetate) the same triacetylglucal as that from acetobromoglucose, which may be taken to indicate that the two acetobromo derivatives have the same ring structure.

[2] Bergmann, M., and Schotte, H., *Ber. chem. Ges.*, **54**, 440 (1921).

[3] Bergmann, M., and Schotte, H., *Ber. chem. Ges.*, **54**, 1564 (1921).

[4] Bergmann, M., *Ann. Chem.*, **434**, 79 (1923).

[5] It is possible that in the normal reaction an oxide is formed between carbon atoms (2) and (3).

EXPERIMENTAL

3-Methylglucal

3-Methyltetracetylglucose.—This substance was prepared in the usual way by the action of acetic anhydride and pyridine on 3-methylglucose. The product was obtained as a colorless syrup which thus far could not be made to crystallize. It analyzed as follows:

5.860 mg. substance: 10.665 mg. CO_2 and 3.215 mg. H_2O.
$C_{15}H_{22}O_{10}$. Calculated. C 49.70, H 6.13.
Found. " 49.63, " 6.14.

Bromo-3-Methyltriacetylglucose.—This substance was prepared from the tetraacetate with hydrogen bromide in acetic acid in essentially the usual way. Again a colorless syrup was obtained which thus far could not be crystallized. However, its bromine content was practically theoretical; being 19.5 per cent whereas the calculated value is 19.98 per cent.

3-Methyldiacetylglucal.—The acetobromo-3-methylglucose was treated with zinc dust[6] in 50 per cent acetic acid in the usual way. Again the product did not crystallize. It was therefore purified by distillation under reduced pressure. At 0.2 to 0.3 mm. pressure it distilled at 125°. In one instance the product obtained in this manner was practically pure glucal on the basis of titration with bromine; in another case, the product titrated for 85 per cent glucal and a second distillation was necessary to obtain an analytically pure product. The yield varied from experiment to experiment. In the best experiment 32.0 gm. of the diacetyl-3-methylglucal were obtained from 100.0 gm. of 3-methylglucose. In another experiment 15.0 gm. were obtained from 60.0 gm. of 3-methylglucose.

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[6] We acknowledge with pleasure our indebtedness to The Sullivan Mining Company for their generosity in furnishing us with zinc dust of high activity.

The substance had the following composition.

5.380 mg. substance: 10.680 mg. CO_2 and 3.235 mg. H_2O.
$C_{11}H_{16}O_6$. Calculated. C 54.07, H 6.61.
Found. " 54.13. " 6.72.

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{-1.32° \times 100}{2 \times 2} = -33.0° \text{ (in chloroform)}.$$

3-Methylglucal.—32.0 gm. of the glucal were added to a solution of 80.0 gm. of barium hydroxide and 475 cc. of water, cooled to 0°. The suspension was shaken in a mechanical shaker until the acetylglucal was completely dissolved. Carbon dioxide gas was passed through the solution and the filtrate from the barium carbonate was evaporated to dryness under reduced pressure. The residue was extracted with alcohol and the alcoholic extract concentrated under reduced pressure to a thick syrup which was reevaporated a few times with benzene. Soon the 3-methylglucal began to crystallize on the walls of the flask in long needles. The substance was dissolved in dry ether from which it crystallized again in long needles. It melted at 62–63° (uncorrected) and had the following composition.

4.890 mg. substance: 9.400 mg. CO_2 and 3.245 mg. H_2O.
$C_7H_{12}O_4$. Calculated. C 52.47, H 7.56.
Found. " 52.42, " 7.42.

The rotation of the substance was

$$[\alpha]_D^{20} = \frac{+0.15° \times 100}{1 \times 1.068} = +14.0° \text{ (in chloroform)}.$$

Conversion of 3-Methylglucal to 3-Methylglucose.—Three experiments were performed, the first of which will be described in detail. 5 gm. of the glucal were dissolved in 50 cc. of water chilled to initial ice formation. To the solution were added 5.0 gm. of benzoic peracid dissolved in chloroform. The mixture was shaken until the aqueous layer no longer decolorized a very dilute aqueous solution of bromine. The aqueous layer was then separated in a separatory funnel, extracted several times with benzene to remove benzoic acid, and finally concentrated nearly to dryness and the distillation continued with benzene. The dry residue was taken up in 25 cc. of 99.8 per cent alcohol. 2.0 cc. of this solution were treated with 1 equivalent of phenylhydrazine in the hope of obtaining a phenylhydrazone but none formed. 5.0 cc. were added to an equivalent of benzylphenylhydrazine but again no crystalline hydrazone formed. The remaining solution was concentrated under diminished pressure nearly to dryness whereupon the substance crystallized. The yield was 1.3 gm. For analysis it was recrystallized from ethyl alcohol (99.8 per cent). The substance melted at 160–162° and had the following composition.

5.530 mg. substance: 8.730 mg. CO_2 and 3.575 mg. H_2O.
$C_7H_{14}O_6$. Calculated. C 43.27, H 7.27.
Found. " 43.05, " 7.23.

The rotation of the substance in water was

Initial.	Equilibrium.
$[\alpha]_D^{20} = \frac{+1.90° \times 100}{1 \times 2} = +95.0°.$	$[\alpha]_D^{20} = \frac{+1.05° \times 100}{1 \times 2} = +52.5°.$

Thus it was evident that the substance was 3-methylglucose. As Bergmann and his coworkers had used the benzoic peracid in ethylacetate solution, two experiments were performed with this solvent. 3.0 gm. of the glucal were used and the yield of crystalline 3-methylglucose was 1.0 gm. in one case and 1.7 gm. in another.

There remained the possibility that the abnormality of the reaction was due to some impurity in the reagent. To exclude this possibility unsubstituted glucal was acted upon by the same benzoic peracid in both chloroform and in ethylacetate solution and in both cases a nearly theoretical yield of mannose was obtained. The phenylhydrazone formed immediately on addition of the equivalent of phenylhydrazine.

Triacetylglucal

Acetoglucal and Benzoic Peracid.—The acetoglucal was prepared in the usual way and recrystallized from alcohol and petrolic ether, giving a product with a rotation of $[\alpha]_D^{15} = -15.1°$ (in 99.8 per cent alcohol).

26 gm. of this acetoglucal were dissolved in 50 cc. of dry chloro-

form and 200 cc. of a 0.54 molal solution of benzoic peracid in chloroform were added (a 10 per cent excess). The mixture was kept at 8–10° for 19 hours and then at room temperature for 5 hours. At the end of this time a sample tested with potassium iodide and acid showed that a little more than the theoretical amount of the benzoic peracid had disappeared. The chloroform solution was shaken repeatedly with sodium bicarbonate solution, washed with water, dried with sodium sulfate, and concentrated under reduced pressure to a syrup. This was taken up in hot isobutyl alcohol from which the product crystallized on standing in the ice box. By concentrating the solution further, two additional crops were obtained. The combined material, 12 gm., was dissolved in 30 cc. of hot isobutyl alcohol and filtered with charcoal. The solution was kept overnight in the ice box, the crystals were filtered off, and washed with petrolic ether-isobutyl alcohol (1:1) and then with petrolic ether. The crystals were fine, colorless needles and the yield of air-dried material was 10 gm. M.p. 139–140°. $[\alpha]^{23}_{D} = +6.5$ (in chloroform). Further recrystallization gave a material with a m.p. of 140.0–140.5° and

$$[\alpha]^{23}_{D} = \frac{+0.50° \times 100}{2 \times 4} = +6.2° \text{ (in chloroform).}$$

No mutarotation was observed in 5 hours. The analysis was as follows:

5.495 mg. substance: 11.200 mg. CO_2 and 2.590 mg. H_2O.
$C_{19}H_{22}O_{10}$. Calculated. C 55.58, H 5.41.
Found. " 55.58, " 5.27.

0.100 gm. of substance shaken with 20.00 cc. of 0.100 N NaOH for 6 hours required 10.10 cc. of 0.100 N HCl for neutralization. Calculated for four acyl groups 9.75 cc. Found, 9.90.

The experiment was repeated but 10 cc. of water were added to the solution of acetoglucal and benzoic peracid in chloroform and the mixture was shaken vigorously in a machine until the reaction was complete (19 hours). The isolation was done in the manner described above. The yield of recrystallized product from 27 gm. of acetoglucal was 9 gm.

The original experiment was repeated, but for the isolation of the product the chloroform solution was concentrated to a thick syrup which was repeatedly extracted with petrolic ether (40–60°). The insoluble residue was taken up in hot isobutyl alcohol. On standing in the ice box, 10 gm. of crystalline product were obtained. After recrystallization from isobutyl alcohol the product melted at 140–141° and had a rotation of

$$[\alpha]_D^{26} = \frac{+0.55° \times 100}{2 \times 4} = +6.9° \text{ (in chloroform).}$$

In order to learn whether the substance was a derivative of mannose or of glucose, 5.0 gm. of recrystallized material were shaken with a saturated (at 0°) solution of dry ammonia gas in dry methyl alcohol, until dissolved. The solution was kept at 2 to 4° for 15 hours and then at room temperature for 4 hours. It was then concentrated under reduced pressure to a thick syrup which was repeatedly extracted with ethyl acetate. The residue was dried, dissolved in water, and diluted to 10.0 cc. A reduction determination (Willstätter) indicated 0.167 gm. of hexose per cc. The rotation, at equilibrium, of 1.0 cc. of the solution diluted to 5.0 cc. with water and measured in a 2 dm. tube (sodium D light) was +3.84°. The rotation, calculated from the reduction value on the assumption that the sugar is glucose, would be $\frac{0.167 \times 52.5° \times 2}{5} = +3.51°$ or if mannose, $\frac{0.167 \times 14.6° \times 2}{5} =$ +0.98°.

On treating the above aqueous solution with phenylhydrazine, no insoluble hydrazone was obtained. On heating with phenylhydrazine and acetic acid, glucosazone resulted, being identified by its melting point and mixed melting point with a pure sample.

Thus it seems that the addition product of the acetoglucal and benzoic peracid is a benzoyl triacetylglucose.

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INTESTINAL NUCLEOTIDASE

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INTRODUCTION

The necessity of using enzymatic hydrolysis of the nucleic acids for the study of their structure has long been realized and earlier work towards this end led to the discovery of enzymes[1] capable of splitting nucleic acid to nucleotides, to nucleosides, or to phosphosugars. In recent years[2] these enzymes have been the object of considerable attention, particularly those classed as nucleotidases (phosphatases) which bring about the cleavage of phosphoric acid from the molecules. This paper deals with this latter type of enzymes which occur in the intestinal secretions.

Nucleotidases are widely distributed in the animal body. However, they are accompanied by other enzymes of which the nucleosidases are the most disturbing inasmuch as such preparations carry the hydrolysis beyond the phase of nucleosides. Fortunately, the intestinal secretion is free of nucleosidases. By virtue of this property, intestinal juice was used successfully for the preparation of nucleosides of the thymus nucleic acid.[3]

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[1] Levene, P. A., and Medigreceanu, F., *J. Biol. Chem.*, **9**, 65, 375, 389 (1911).

[2] See for example: Levene, P. A., Yamagawa, M., and Weber, I., *J. Biol. Chem.*, **60**, 693 (1924). Levene, P. A., and Weber, I., *J. Biol. Chem.*, **60**, 707, 717 (1924). von Euler, H., and Brunius, E., *Ark. Kemi Mineral. Geol.*, **9**, 1 (1927); *Ber. chem. Ges.*, **60**, 1584 (1927). Martland, M., and Robinson, R., *Biochem. J.*, **21**, 665 (1927); **23**, 237 (1929). Deutsch, W., *Z. physiol. Chem.*, **171**, 266 (1927). Erdtmann, H., *Z. physiol. Chem.*, **172**, 182 (1927); **177**, 211, 231 (1928). Akasawa, *J. Biochem.*, Japan, **10**, 157 (1928). Levene, P. A., and London, E. S., *J. Biol. Chem.*, **81**, 711 (1929); **83**, 793 (1929). Hommerberg, C., *Z. physiol. Chem.*, **185**, 123 (1929). Deutsch, W., and Rösler, K., *Z. physiol. Chem.*, **185**, 146 (1929). Deutsch, W., and Laser, R., *Z. physiol. Chem.*, **186**, 1 (1929). Tannhauser, S. J., and Angermann, M., *Z. physiol. Chem.*, **186**, 13 (1929).

Thus a nucleotidase free of nucleosidase can be prepared; not however, a nucleosidase free of nucleotidase, and such an enzyme is needed for the preparation of ribophosphoric and of desoxyribophosphoric acids.

With a view of accomplishing the separation of the two enzymes a systematic study of the properties of nucleotidase was undertaken. The present paper deals with the question of the specificity of intestinal nucleotidase. In order to study this specificity, a comparative study was made of the action of the enzyme on a series of phosphoric esters as follows:

1. Glycerol phosphate (used as a standard for enzyme activity).
2. Phospho-sugars.
    (*a*) Hexosemonophosphate from α-diacetonefructose.
    (*b*) " " β-diacetonefructose.
    (*c*) Neuberg monophosphate.
    (*d*) Hexosediphosphate.
    (*e*) " methylglucoside.
    (*f*) Hexosemonophosphate from monoacetoneglucose.
    (*g*) " " diacetoneglucose.
3. Nucleotides.
    (*a*) Adenilic acid.
    (*b*) Inosinic "
    (*c*) Uridine phosphoric acid.
4. Nucleic acids.
    (*a*) Yeast nucleic acid.
    (*b*) Thymus nucleic acid.

The substrate concentrations were in general 0.161 molal in order to permit the isolation of the products of hydrolysis if desired. However, in the case of thymus nucleic acid this condition was a handicap inasmuch as the substrate or some hydrolytic product inhibits the reaction considerably at this concentration.

The hydrolyses were carried out at a pH of 8.6 to 8.7, this value being slightly below the optimum pH of 9.0 to 9.2. Since the dissociation constants of phosphoric acid, glycerol phosphate, and hexosephosphates are nearly the same[4] there was no change in pH on hydrolysis[5] and hence for these substrates no buffers were necessary. Likewise, none was required for the nucleotides, but for the nucleic acids suitable buffers, namely diethylbarbiturate, borate, and acetate, were used.

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[3] Levene, P. A., and London, E. S., *J. Biol. Chem.*, **81,** 711 (1929); **83,** 793 (1929).

[4] Meyerhoff, O., *Naturwissenschaften,* **14,** 1277 (1926).

The initial rates of hydrolysis of all of the substances studied are given in Table I. For purposes of comparison, the rates in this table were calculated to correspond to an arbitrary enzyme concentration of 2 activity units[6] per cc. of substrate solution.

TABLE I

Comparative Rates of Hydrolysis

Sample No.	Substance.	Initial rate, mg. P per cc. per hr.	Relative rate.
1	Glycerol phosphate	2.20	1.00
2 b	Hexosemonophosphate from β-diacetonefructose	2.20	1.00
2 e	Hexosediphosphate methylglucoside	2.00	0.91
2 d	Hexosediphosphate	1.96	0.89
2 f	Hexosemonophosphate from monoacetoneglucose	1.94	0.88
2 c	Neuberg monophosphate	1.90	0.86
2 g	Hexosemonophosphate from diacetoneglucose.	1.54	0.69
2 a	" " α-diacetonefructose	1.40	0.64
3 c	Uridine phosphoric acid	1.78	0.81
3 a	Adenilic acid	1.30	0.59
3 b	Inosinic "	0.80	0.36
4 a	Yeast nucleic acid	0.15	0.07
4 b	Thymus " "	0.10	0.05

The hydrolyses were nearly complete in 6 to 12 hours except in the case of the nucleic acids. Complete hydrolysis of the latter compounds could not be accomplished even by adding additional portions of enzyme. It is interesting to note that the nucleotides hydrolyze nearly as rapidly as the sugar esters but that the nucleic acids hydrolyze much more slowly. Uridine phosphoric acid is more rapidly split than adenilic acid and this latter in turn more readily than inosinic acid. This order is different from that in the case of hydrolysis with acid, where adenilic acid is split more rapidly than either uridine phosphoric acid and inosinic acid (the rates of the last two being nearly the same). It is also seen that with regard to the intestinal enzyme, thymus nucleic acid is somewhat more resistant than yeast nucleic acid. The rates of hydrolysis of hexosediphosphate and hexosediphosphate methylglucoside are exactly the same. No reducing sugars could be detected in the hydrolysates of hexosediphosphate methylglucoside or of the nucleotides, as was expected because of the absence of nucleosidases[7] in the enzyme preparations used.

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[5] When 0.016 molal glycerol phosphate (*i.e.*, 0.1 the usual concentrations) was used, a slight increase in pH was observed (∽0.2 to 0.3 pH) which is in agreement with the results published recently by Hommerberg. (Hommerberg, C., *Z. physiol. Chem.*, **185**, 123 (1929).) This author used exceedingly dilute solutions of glycerol phosphate (0.001 to 0.007 molal) and he found that the pH increased by nearly 1.0 unit during hydrolysis. The increase, however, cannot be due to the reason ascribed to it by this author, namely to the liberation of free phosphoric acid, for the reason indicated above.

[6] For definition, see p. 180.

Instead of using the intestinal juice directly as in a previous work[3] the enzyme precipitated from its solutions with acetone was used. Different samples thus obtained hydrolyzed a standard glycerol phosphate solution at approximately the same rate. In addition, these samples showed a constant parallelism in their capacity for hydrolyzing both nucleotides and thymus nucleic acid. However, one sample showed an exceptional behavior. This sample had an activity towards glycerol phosphate slightly higher than the average and likewise hydrolyzed adenilic acid with the same high rate but hydrolyzed nucleic acid to phosphoric acid and to nucleosides very slowly, the initial rate being only one-tenth of the usual value. After 24 hours the extent of the hydrolysis was 17 per cent instead of 60 per cent. This sample had a distinctly green color instead of the customary yellow one, thus indicating the presence of bile products to which possibly the peculiar behavior of this sample may be attributed. It is equally conceivable that only slight hydrolysis occurred because of the absence of nucleinase, an enzyme converting polynucleotides into the component mononucleotides. If the latter explanation is correct, it may be assumed further that degradation of nucleic acids to nucleotides must precede the dephosphorylation, that is, the phosphatase is capable of dephosphorylating nucleotides only, and not nucleic acids. This suggestion has already been made. Indeed, it was found that thymus nucleic acid was split more rapidly to nucleotides with the normal enzyme than with the abnormal sample.

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[7] Levene, P. A., and Medigreceanu, F., *J. Biol. Chem.*, 9, 375 (1911).

On this assumption it is unnecessary to postulate the existence of more than one phosphatase.

The addition of magnesium ion in order to remove the inhibition by the phosphate ion liberated in the hydrolysis of thymus nucleic acid gave results that were not as satisfactory as those carried out in the absence of magnesium ion. It may be that the inhibition by the magnesium ion and the ammonium acetate outweighs the advantage gained by the removal of the phosphate. The hydrolysis curve, as will be noted, is quite unlike the usual curves but the per cent hydrolysis finally reached the normal value.

EXPERIMENTAL

A. Standard of Phosphatase Activity.—As a basis for the assay of the enzyme activity of the samples the hydrolysis of a standard sodium glycerol phosphate solution was used. This substrate is very convenient for this purpose since the hydrolysis may be readily followed directly on the hydrolysate.

This glycerol phosphatase activity is probably a true measure of the *general phosphatase activity* of the samples, since the capacity of the enzyme for hydrolyzing the different types of organic phosphates (such as the phospho-sugars and nucleotides, but with the exception of the nucleic acids) seems to be proportional to their capacity for hydrolyzing glycerol phosphate.

The glycerol phosphate standard solution was prepared by dissolving crystalline sodium glycerol phosphate (Eastman Kodak Company) in distilled water, the solution being made 0.322 molal on the basis of the phosphorus in the crystalline material. The solution was brought to a pH of 8.6, was protected with toluene, and was kept in the cold. The procedure in the assay was as follows: to 10.0 mg. of enzyme were added 2.5 cc. of water and, after thorough shaking, 2.5 cc. of this standard glycerol phosphate solution. The solution was placed in a thermostat at 30°, and phosphate ion determinations were made at convenient time intervals according to the method indicated later for the phospho-sugars.

It was found convenient to adopt as a unit of activity the amount of enzyme capable of liberating from the glycerol phosphate substrate, 1.0 mg. of phosphorus (as phosphate ion) in the course of the 1st hour of the hydrolysis. This amount of phosphorus corresponds to a hydrolysis of 20 per cent since a 0.161 molal solution contains 5.0 mg. of phosphorus per cc. The activity coefficients are then given by dividing this initial rate by the weight of solid enzyme expressed in mg. per cc.

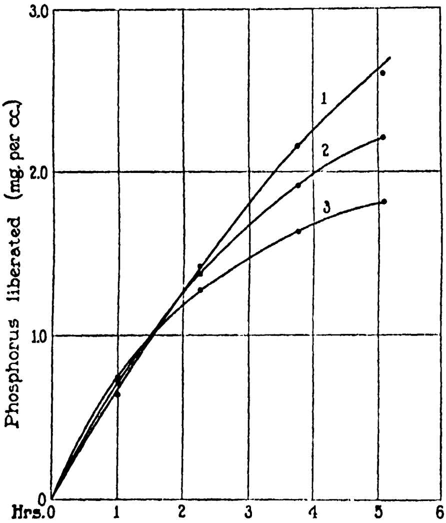

FIG. 1. Effect of glycerol phosphate concentration. Enzyme concentration = 1.0 mg. per cc. The glycerol phosphate concentration was for Curve 1, 0.322 M; Curve 2, 0.161 M; Curve 3, 0.080 M.

Within a limited range of concentrations the initial rates of hydrolysis are independent of the substrate concentration (Fig. 1) but are proportional to the enzyme concentration (Fig. 2).

B. The Enzyme.—The intestinal secretions of dogs collected from an intestinal fistula were the source of the crude enzyme material used. These solutions varied considerably as to amount, activity, and opacity. The solutions as obtained had a pH of 7.0 and were usually light yellow in color, though in some cases were distinctly green due perhaps to bile products. The solutions were filtered through cheese-cloth, centrifuged, and then poured into 10 volumes of acetone. The precipitate was quickly centrifuged, washed once with acetone, twice with anhydrous ether, and put into a vacuum desiccator to dry over phosphorus pentoxide and paraffin. The amount of precipitate obtainable in this way ranged from 5 to 10 mg. per cc. of solution but could be practically doubled by not centrifuging so quickly after the precipitation. The material obtained by more complete precipitation was not so satisfactory since it was not as stable nor as active as that obtained by more rapid work. The crude dry enzymes thus obtained were fluffy powders and possessed the characteristic color of the solution from

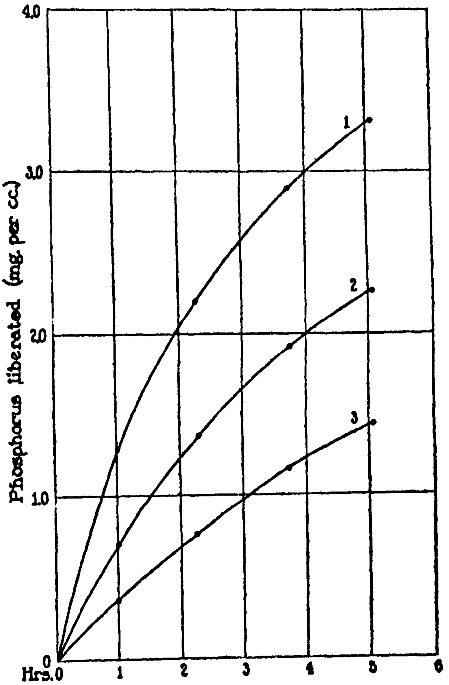

FIG. 2. Effect of enzyme concentration. Glycerol phosphate concentration = 0.161 M.

Enzyme concentration.	Initial rate of hydrolysis.
Curve 1, 2.0 mg. per cc.	1.40 mg. P per cc. per hr.
" 2, 1.0 " " "	+0.69 " " " " " "
" 3, 0.5 " " "	+0.35 " " " " " "

which they were precipitated except in a few cases where light yellow solutions yielded almost white samples. They were in general very stable and usually had activity coefficients between 0.6 and 0.8, though occasionally samples as high as 1.0 and as low as 0.5 were obtained. The dry enzyme samples had an inorganic phosphate content equivalent to about 3 per cent of phosphorus, there evidently being no organic phosphates present since no liberation of phosphorus was observed when they were allowed to stand in solution. It may be mentioned here that the original enzyme solutions when kept at 0° and protected with a small amount of toluene, also maintained their glycerol phosphatase activity unchanged for as long as a month. However, the character, amount, and activity of the precipitate prepared from solutions which were allowed to stand for some time, did not appear to be as satisfactory as those prepared from fresh enzyme solutions.

TABLE II

Enzyme Activities

Material.	Activity units.	Activity coeffi-cient (units per mg.).	Total activity recov-ered per cc. of solution.
Original enzyme solution; dry weight, 23.7 mg. per cc.	1.45 per cc.	0.061	(1.45)
Acetone-precipitated enzyme			
(*a*) Usual method of precipitation. Yield 5.84 mg. per cc.	1.96 per 2 mg.	0.98	5.72
(*b*) Maximum precipitation. Yield 11.1 mg. per cc.	1.00 per 2 mg.	0.50	5.55

By the simple acetone precipitation considerable purification of the enzyme was obtained, the activity coefficient increasing about 15-fold. This is shown in Table II. It also shows the effect of the method of purification adopted as compared to that of more complete precipitation. Thus, by approximately doubling the amount of precipitate the activity coefficient was halved, hence the total activity recovered was not increased. The fact that there was a greater activity in the precipitates than was apparently present in the original solution is probably due to inhibiting substances contained in the latter.

C. Methods. Hydrolyses and Analyses.—The solutions of sodium salts of the phosphate substrates were prepared differently according to the form in which the material was available. The origin of these samples will be indicated under the separate hydrolysis experiments. In the case of sodium salts, they were dissolved directly in distilled water to the correct volume and adjusted to the desired pH. In the case of the free phosphoric acid esters they were dissolved in dilute alkali to the correct volume and pH. In the case of the barium salts they were added to water, the calculated quantity of sodium carbonate solution was added slowly with stirring, the solutions centrifuged, and the filtrates used after testing for the absence of barium with a trace of sulfate, and bringing them to the correct pH. The concentrations were usually such that on complete hydrolysis there would be liberated 5.0 mg. of phosphorus per cc. of hydrolysate, that is 0.161 molal (diphosphates, 0.080 molal). Values differing from 5.0 mg. per cc. are indicated in the legends of the figures. Such values are placed in parentheses following the name of the substrate. In the case of thymus nucleic acid it was found better to use one-third of this concentration, though solutions containing up to 6 per cent of this nucleic acid were sometimes used.

The enzyme concentrations were 2 mg. per cc. of substrate except in the case of the nucleic acids where higher and variable amounts (from 9 to 15 mg. per cc.) were used. Weighed amounts of enzyme were added directly to definite volumes of the substrates.

The hydrolyses were carried out in a thermostat maintained at 30°. The samples were in all cases protected from bacterial infection by a small amount of toluene, a substance found to have no effect on the hydrolysis rates. Both chloroform and sodium arsenate showed an inhibition. In some cases octyl alcohol was found useful as an antifroth in preparing the substrate solutions. It was interesting to find that it had a slight enhancing effect on the enzyme. This was indicated by the fact that the hydrolysis of glycerol phosphate in the presence of a few drops of this material was slightly more rapid than in its absence. The substrates were hydrolyzed at a pH of 8.6 to 8.7 and in most cases they maintained this value throughout the complete hydrolysis by virtue of their own buffering capacity at this pH. For the exception to this, the nucleic acids, buffers of diethylbarbiturate, borate, and acetate were used.[8] The buffer solutions were used in the hydrolyses in the ratio of 1 cc. of buffer to 2 cc. of the nucleic acid substrate solution. The buffering capacity even under these conditions was not quite sufficient to prevent completely a change in the pH during the hydrolysis; small amounts of alkali were added occasionally, in order to maintain the pH within the range of 8.3 to 8.7.

The rate measurements were made by determining colorimetrically the free phosphate ion concentration. The method of Kuttner and Cohen[9] was used. Nearly all of the experiments were made in duplicate. Initial determinations of the phosphate were always made and this value which was quite variable, though small, was corrected for in the curves which are given. Suitable samples were removed at convenient time intervals from the hydrolysates and treated differently according to the type of substrate. For the phospho-sugars, 0.5 cc. of the hydrolysate was pipetted into 4.5 cc. of 1 per cent trichloroacetic acid which was then filtered with the aid of a small amount of kieselguhr through an ordinary filter. This solution was diluted so as to be approximately equivalent to the standard containing 0.025 mg. of phosphorus per cc. 1 cc. samples were then compared with the standard. In the case of the nucleotides and the nucleic acids the procedure was necessarily modified since the determinations could not be made directly on the trichloroacetic acid filtrates. The nucleotides did not precipitate with the trichloroacetic acid and interfered in the colorimetric method. The nucleic acid filtrates could not be clarified nor could the nucleic acid be completely precipitated. This later caused interference. For the nucleotides 0.5 cc. samples of the hydrolysates were added to 2 cc. of water and 1 cc. of magnesia mixture.[10] To this, a drop of dilute ammonia was immediately added and the solution stirred thoroughly. The magnesium ammonium phosphate precipitated in this way was always crystalline. A minute later 1 cc. of concentrated ammonium hydroxide (sp. gr. 0.90) was added. Because the enzymatic hydrolysis continued with an appreciable rate even in these strong ammonia solutions the precipitates were allowed to stand only 10 to 15 minutes, after which time they were filtered through a micro filter and washed with 2 cc. of 2 per cent ammonium hydroxide. The precipitates were redissolved in 10 cc. of 0.5 N HCl, made up to 25 cc., diluted if necessary, and determined as usual. Actually, under the conditions employed the precipitation of the phosphate is essentially complete even in this short interval of time.

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[8] The veronal buffer of pH 8.6 was made according to the directions of Michaelis (Michaelis, L., *J. Biol. Chem.*, **87**, 33 (1930)). The borate buffer was the standard Clark and Lubs' (Clark, W. M., The determination of hydrogen ions, Baltimore (1928)) boric acid-potassium chloride-sodium hydroxide buffer of pH 8.6. The acetate buffer was prepared by dissolving 38.5 gm. (0.5 mol) of ammonium acetate in 13.5 cc. of 5 N ammonium hydroxide and diluting to 500 cc.

[9] Kuttner, T., and Cohen, H. R., *J. Biol. Chem.*, **75**, 517 (1927).

[10] 1 liter of the magnesia mixture used contained 75 gm. of magnesium chloride (6 mols of water) and 100 gm. of ammonium chloride.

In the nucleic acid experiments prior to precipitating the phosphate the unchanged nucleic acid was removed. This was accomplished by pipetting 5 cc. samples of the hydrolysate into an equal volume of 10 per cent trichloroacetic acid, adding some kieselguhr, and filtering with suction. The filtrate, which was still turbid, became nearly clear when ammonia was added until slightly alkaline. After a short time the remaining turbid material coagulated and could be removed easily by filtering with an ordinary filter. To the filtrate (about 70 cc.) 3 to 5 cc. of magnesia mixture were added and the precipitate allowed to become well formed before 12 cc. of concentrated ammonia were added. The precipitate was allowed to stand overnight, was filtered off in a small Gooch funnel, dissolved in 15 cc. of 1 N HCl, and the phosphate determination performed as usual.

In order to avoid the inhibition effect of the phosphate[11] it was desirable to carry out a hydrolysis in which the phosphate was removed as fast as formed. The method was similar to that reported.[12] The nucleic acid was dissolved in magnesia mixture[13] instead of in water, the solution was allowed to stand overnight, and filtered. The filtrate was used with the acetate buffer in the usual manner. The phosphate which precipitated during the reaction was readily dissolved by 10 per cent trichloroacetic acid, so no modification of the phosphate analysis was necessary.

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[11] See Hommerberg, C., *Z. physiol Chem.*, **185**, 130 (1929).

[12] Deutsch, W., *Z. physiol. Chem.*, **186**, 11 (1929). The amount of magnesium ion used in the hydrolysis experiment recorded there is obviously insufficient for the precipitation of the phosphate at the end of the hydrolysis.

[13] 1 liter of the magnesia mixture used contained 57 gm. of magnesium acetate (4 mols of water), 180 gm. of ammonium acetate, and 60 cc. of a 10 per cent ammonium hydroxide solution. The solution was 0.25 molal in respect to Mg^{++} and had a pH of 8.6.

D. Hydrolysis of Phospho-Sugars.—The barium salts of the phosphoric acid esters from α- and β-diacetonefructose are those previously prepared.[14] The barium salt of the Neuberg ester was prepared according to the method of Neuberg.[15] The substrates for the hydrolyses were made from these samples as indicated above. The hydrolysis curves are shown in Fig. 3. The ester from β-diacetonefructose hydrolyzed at exactly the same rate as glycerol phosphate, whereas the ester from α-diacetonefructose

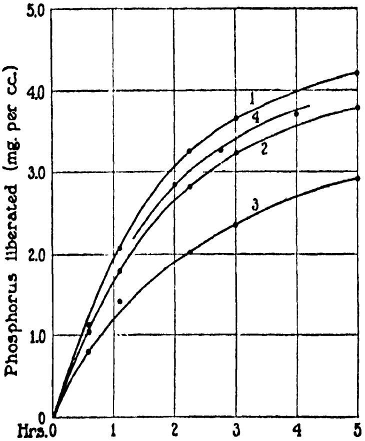

Fig. 3.

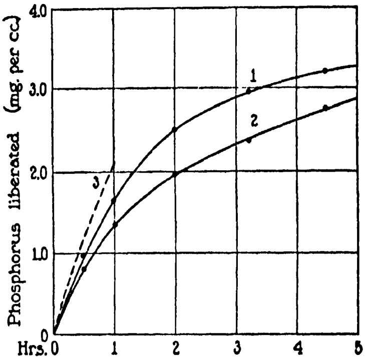

Fig. 4.

Fig. 3. Hydrolysis of fructose phosphoric esters. Curve 1, glycerol phosphate standard; hexosemonophosphate from β-diacetonefructose; Curve 2, Neuberg monophosphate; Curve 3, hexosemonophosphate from α-diacetonefructose; Curve 4, hexosediphosphate and the corresponding methylglucoside.

Fig. 4. Hydrolysis of glucose phosphoric esters. Curve 1, hexosemonophosphate from monoacetoneglucose (4.5 mg. P per cc.); Curve 2, hexosemonophosphate from diacetoneglucose; Curve 3, glycerol phosphate standard.

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[14] Raymond, A. L., and Levene, P. A., *J. Biol. Chem.*, **83**, 619 (1929).

[15] Neuberg, C., *Biochem. Z.*, **88**, 432 (1918).

hydrolyzed more slowly. The Neuberg monophosphate has a hydrolysis rate approaching that of the ester from β-diacetonefructose. Thus the rate of hydrolysis of a phospho-sugar varies greatly with the position occupied by the phosphoric acid radical.

The hexosediphosphate barium salt was prepared from the commercial calcium salt through the intermediary of the sodium salt. The methylglucoside of this hexosediphosphate obtained as the barium salt is that previously described.[16] The hydrolysis curves for the two substances were practically identical and were midway between the curves (Fig. 3) for the ester from β-diacetonefructose and the Neuberg ester.

The hexosemonophosphates from monoacetone-[17] and diacetoneglucose[14] were isolated as barium salts after having been purified as brucine salts. The hydrolysis curves of the substrates prepared from these samples are given in Fig. 4. The monophosphate obtained from monoacetoneglucose hydrolyzed at the same rate as the Neuberg ester. The rate of hydrolysis of the monophosphate from diacetoneglucose was lower than that of the preceding monophosphate, its rate being approximately the same as that of the phosphoric acid ester from α-diacetonefructose.

E. Hydrolysis of Nucleotides.—Two purine derivatives, adenilic acid and inosinic acid were hydrolyzed. The former was obtained as the free crystalline acid and the latter as the barium salt. The pyrimidine nucleotide, uridine phosphoric acid, was obtained as the barium salt. The hydrolysis curves of the substrates prepared from these samples are given in Fig. 5. The pyrimidine nucleotide, uridine phosphoric acid, is more rapidly split than the purine nucleotides, its rate being slightly lower than that of the Neuberg ester. Of the two purine nucleotides, adenilic acid is more rapidly split than inosinic acid, the rate of the first being the same and that of the second being lower than that of the phosphoric acid ester from α-diacetonefructose.

For a comparison, the hydrolysis curve of yeast nucleic acid is plotted in Fig. 5 with those of the nucleotides. The comparison is only qualitative since the enzyme concentration in the hydrolysis of the nucleic acid was 4.5 times that used in the case of nucleotides. The contrast between the rates of hydrolysis of the nucleotides and the nucleic acids is indicated more strikingly in Table I, where the comparative rates are given for a standard amount of enzyme.

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[16] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **80**, 633 (1928).

[17] Prepared by phosphorylation of monoacetoneglucose and subsequent hydrolysis.

F. Hydrolysis of Nucleic Acids.—Yeast nucleic acid, as the free acid, and thymus nucleic acid, as the semi-crude sodium salt and as purified free acid, were used as substrates. Various curves of the hydrolysis of these materials are given in Figs. 6 to 10.

In Fig. 6 is shown a comparison of the rates and the extents of hydrolysis of yeast and thymus nucleic acids. The former hydrolyzes faster and reaches a greater per cent hydrolysis than does the latter, showing the thymus nucleic acid to be somewhat more stable than yeast nucleic acid, though the difference between the two does not appear to be as great as would be supposed from previous indications.[1, 3]

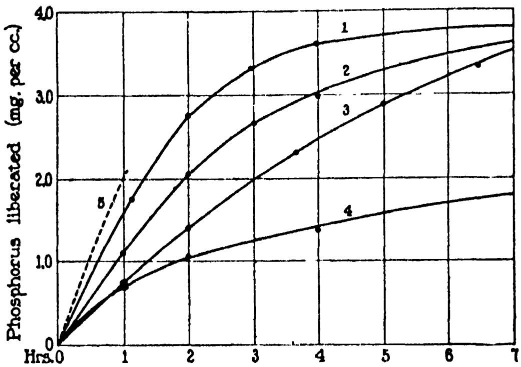


FIG. 5. Hydrolysis of nucleotides. Curve 1, uridine phosphoric acid (3.9 mg. P per cc.); Curve 2, adenilic acid (4.1 mg. P per cc.); Curve 3, inosinic acid; Curve 4, yeast nucleic acid; Curve 5, glycerol phosphate standard.

The effect of the buffers on the rate of hydrolysis of thymus nucleic acid is given in Fig. 7. The acetate buffer inhibits the hydrolysis of the glycerol phosphate standard to the extent of about 20 per cent, whereas the other two buffers show no effect on this hydrolysis. It is probable that the acetate buffer also inhibits the nucleic acid hydrolysis by the same amount and taking this into account by using a higher concentration of enzyme with this buffer, exactly similar hydrolysis curves were obtained with the acetate and veronal buffers. However, the borate buffer had a

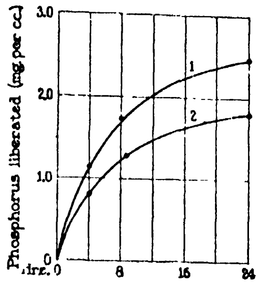


FIG. 6.

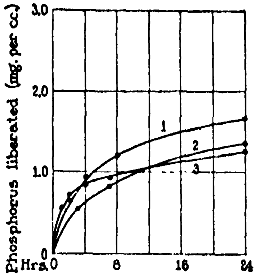
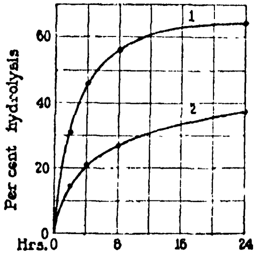


FIG. 7.

FIG. 6. Hydrolysis of yeast and thymus nucleic acids. Curve 1, yeast nucleic acid (5.0 mg. P per cc.). Maximum value reached = 3.8 mg. P per cc.; Curve 2, thymus nucleic acid (4.0 mg. P per cc.). Maximum value reached = 2.5 mg. P per cc.

FIG. 7. Hydrolysis of thymus nucleic acid. Effect of buffers: Curve 1, with veronal or acetate buffer; Curve 2, with acetate buffer and Mg^{++}; Curve 3, with borate buffer.



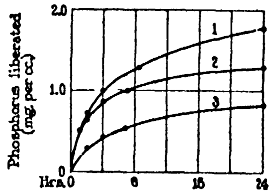


FIG. 8. FIG. 9.

FIG. 8. Hydrolysis of thymus nucleic acid. Effect of substrate concentration: Curve 1, 2 per cent nucleic acid solution (1.3 mg. P per cc.); Curve 2, 6 per cent nucleic acid solution (4.0 mg. P per cc.).

FIG. 9. Hydrolysis of thymus nucleic acid. Effect of purification: Curve 1, unpurified and satisfactorily purified samples (4.0 mg. P per cc.); Curves 2 and 3, unsatisfactorily purified samples (4.0 mg. P per cc.).

peculiar effect on the nucleic acid hydrolysis, though it showed no effect on the hydrolysis of glycerol phosphate. For this buffer the hydrolysis curves showed a marked break between 15 to 20 per cent hydrolysis though the rates were entirely normal up to this point. This effect is perhaps due to the combination of some partially hydrolyzed portion of the nucleic acid with boric acid. The decrease in hydrolysis rate brought about by the addition of magnesium ion to the reaction mixture is also indicated in Fig. 7.

In the hydrolysis of the phospho-sugars and the nucleotides, the substrate concentrations were 0.161 molal. In these cases the hydrolysis was always complete after a sufficient length of time (12 to 24 hours). On the other hand the hydrolysis of the nucleic acids at this concentration could not be made complete even by adding additional portions of enzyme and allowing the hydrolysis to continue for a number of days. It was found advantageous to use a more dilute solution of these substrates since both the rate and the extent of hydrolysis could be materially increased as shown in Fig. 8, though complete hydrolysis was not accomplished even in these dilute solutions.

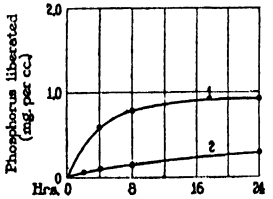


FIG. 10. Hydrolysis of thymus nucleic acid. Effect of enzyme sample: Curve 1, average sample—phosphatase activity = 1.7 (1.3 mg. P per cc.); Curve 2, abnormal sample—phosphatase activity = 2.0 (1.3 mg. P per cc.).

The preliminary hydrolyses of thymus nucleic acid were carried out on a semi-crude sample. Later it was desired to use better material but in several cases the purification of the thymus nucleic acid yielded samples which had much lower rates of hydrolysis than the unpurified sample. While the chemical analysis of these slowly hydrolyzed samples indicated that they were not as pure as desired, the differences from the correct analysis values were not as great as might be expected from the deviations noted in the rates of hydrolysis. These samples possibly contained some diphosphoric esters of pyrimidine nucleosides. Careful purification yielded samples which analyzed perfectly and hydrolyzed with the same rate as the unpurified sample. A comparison of these various samples can be made from Fig. 9.

The anomolous activity of the enzyme sample mentioned in the introduction is indicated in Fig. 10. Curve 1 represents the hydrolysis of thymus nucleic acid with a normal sample of the enzyme which, as indicated in the previous discussion, shows a high rate of hydrolysis with both glycerol phosphate and adenilic acid. Curve 2 represents the hydrolysis with the abnormal sample which also hydrolyzes glycerol phosphate and adenilic acid with the same high rate as the normal sample. A more striking comparison is obtained from Table III which gives the initial rates of hydrolysis of the three types of substrates with the two different enzyme samples.

TABLE III

Comparative Rates of Hydrolysis

Substrate.	Initial rate of hydrolysis.			
	With normal sample.		With abnormal sample.	
	Mg. P per cc. per hr.	Relative rate.	Mg. P per cc. per hr.	Relative rate.
Glycerol phosphate	1.65	1.00	2.03	1.23
Adenilic acid	0.95	0.58	1.06	0.64
Thymus nucleic acid	0.25	0.16	0.022	0.013

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STUDIES IN POLYMERIZATION AND CONDENSATION

VI. 5,6-DIHYDROXYHEXANONE-2

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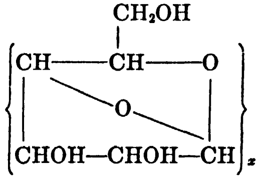
(Received for publication, July 15, 1930)

In a previous communication[1] a report was made on the spontaneous condensation of dihydroxyacetone into dihydroxyacetonyldihydroxyacetone and di-(dihydroxyacetonyl)dihydroxyacetone. It seemed desirable to extend the observations to simple dihydroxyketones in which the positions of the two hydroxyls were on carbon atoms 5 and 6 for the reason that such an arrangement would permit <2, 5> and <2, 6> lactal formation and hence present conditions similar to those existing in common sugars. The investigation seemed all the more desirable for the reason that Hibbert and Timm[2] had already studied one condensation product of 5,6-dihydroxyhexanone-2 and had attributed to it a structure analogous to that which Hibbert had postulated for cellulose.



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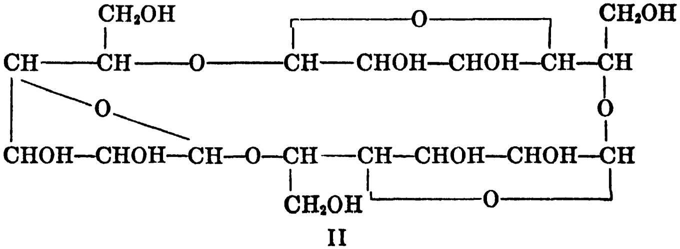

II

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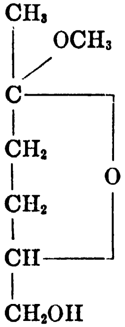
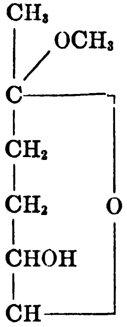
[1] Levene, P. A., and Walti, A., *J. Biol. Chem.*, **78**, 23 (1928).

[2] Hibbert, H., and Timm, J. A., *J. Am. Chem. Soc.*, **45**, 2433 (1923).

However, before entering into the study of the polymerization or condensation of 5,6-dihydroxyhexanone-2, it was desirable to obtain more information on the structure of the substance itself, particularly with respect to its cyclic nature. In the homogeneous state the substance probably consists of the two forms in equilibrium. In some of its derivatives, however, the cyclic form apparently predominates. Thus, on treatment with methyl alcohol containing 0.5 per cent of hydrogen chloride, a methylcyclo derivative is obtained, having one of the following structures.



III

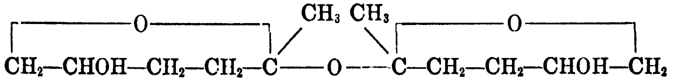


IV

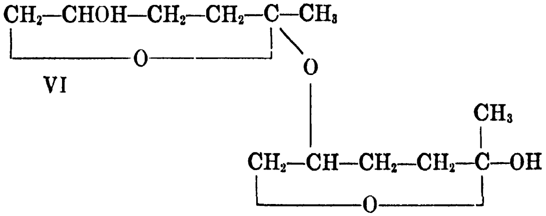
The molecular refraction of the substance is 36.97 and the calculated value is 37.13. In harmony with the view of the cyclic structure were also the results of further methylation and of acetylation inasmuch as the methylcyclo derivative gave a monoacetyl and a monomethyl derivative.

Condensation of 5,6-Dihydroxyhexanone-2

Freshly distilled ketone is a mobile colorless liquid. Often, on standing at 25° and sometimes even on standing at 0°, as much as 50 per cent of the original ketone undergoes condensation into a substance distilling at 175–180° at 0.2 to 0.3 mm. pressure and having the composition of a desoxydisaccharide, dihydroxyhexanonyldihydroxyhexanone. As such, the substance gave a diacetyl derivative. However, after treatment at room temperature with dry methyl alcohol containing 0.5 per cent of hydrogen chloride the disaccharide was apparently hydrolyzed with the formation of methylcyclodihydroxyhexanone-2. The great instability of the desoxydisaccharide is not surprizing in view of the fact that 5,6-dihydroxyhexanone-2 is a desoxy sugar similar to glucodesose and in view of our present knowledge of the great instability of the glucosides of this group of substances. Because of the great instability of this disaccharide, it is difficult to choose between the two structures V and VI which it may possess; however, structure V is the more probable one.



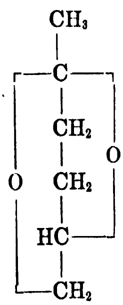
V



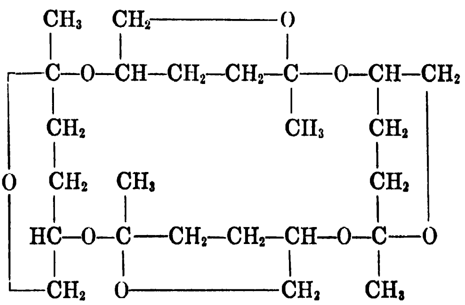
VI

Polymerization Product Obtained from 5,6-Dihydroxyhexanone-2 with Sulfuric Acid as Catalyst (Hibbert and Timm)

In the light of the above result on the spontaneous formation of the disaccharide, it became necessary to reinvestigate the structure of the substance prepared by Hibbert and Timm[2] which according to these authors should have one of the following structures in analogy with the structure postulated by Hibbert for cellulose.



VII

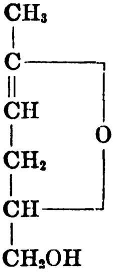


VIII

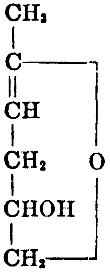
As it is seen from figures VII and VIII, substances of this structure have no free hydroxyls and therefore do not permit the formation of acetyl derivatives. In reality, however, the substance prepared by us according to Hibbert and Timm formed an acetyl derivative containing one acetyl group for each dihydroxyketone radical and yet the substance had the composition of an anhydrodihydroxyhexanone, in agreement with the analytical data of Hibbert and Timm. In order, therefore, to explain the structure of the substance of Hibbert and Timm, it was necessary to obtain more information on the structure of anhydro-5,6-dihydroxyhexanone-2.

Anhydride of 5,6-Dihydroxyhexanone-2

Theoretically the anhydride may be formed either through a second ring formation similar to that observed in the case of glucosans (structure VII) or through desaturation (structures IX or X).



IX



X

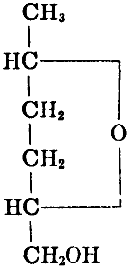
Indeed, the existence of the two forms (VII and IX or VII and X) could be easily demonstrated. On heating 5,6-dihydroxyhexanone-2 to 150° for 9 hours there occurred a loss of water. The lower boiling fraction of this product consists apparently of the anhydride of structure VII on the basis of the values of the molecular refraction and of the parachor. On the other hand, if the condensation product is acetylated prior to distillation and the acetylation product is distilled, then a substance is obtained with the composition of a monoacetyl derivative of the anhydrodihydroxyhexanone. Its structure is seen from the fact that it contains an acetyl group and furthermore from the fact that in the presence of Adams' catalyst, this substance absorbs hydrogen in an amount corresponding to one double bond. Also the determined values of the molecular refraction and of the parachor are in agreement with the calculated ones for the acetyl derivative of substances IX or X.

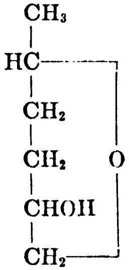
Polymerization of the Anhydro-5,6-Dihydroxyhexanone-2

At times, a hard, slightly brownish resin was obtained on heating the 5,6-dihydroxyhexanone-2 at 150° for 10½ hours with interruptions after each 3½ hour period. This material on heating under reduced pressure and at a temperature not exceeding 230° lost only water and the residue had the composition of anhydrodihydroxyhexanone. On acetylation, this polymerized product formed an acetyl derivative which had the composition of a monoacetyl derivative of an anhydrodihydroxyhexanone, thus showing that the hydroxyl of the anhydride is not involved in the polymerization. On the other hand, on standing at room temperature in methyl alcohol containing 0.5 per cent of hydrogen chloride, the substance remained unchanged; on refluxing with the same solution, the substance was methylated only to the extent of one methyl group to about five of the ketone. It is not excluded that under these conditions a slight depolymerization took place. Also, the absorption of hydrogen in the presence of Adams' platinum dioxide catalyst was very small, approximately 1 molecule of the gas being absorbed for every 7 molecules of the anhydride. All these properties seem to indicate that the double bond is involved in the polymerization. Thus, it seems to us that the 5,6-dihydroxyhexanone-2 cannot serve as a model of a true sugar and its polymerization product cannot serve as a model of a polysaccharide. Rather is 5,6-dihydroxyhexanone-2 a model of a desoxy sugar and the resin formation which so readily takes place a model of the events in the 2-desoxy sugars. Indeed, the tendency of the latter to polymerize with tar formation is one of their principal characteristics. *It is possible that the mechanism of polymerization of desoxyglucoses is analogous to the mechanism of polymerization of 5,6-dihydroxyhexanone-2 which takes place in two steps; namely, anhydride formation giving rise to a double bond which then causes the new substance to polymerize. The same is undoubtedly the process of formation of the substance of Hibbert and Timm.*

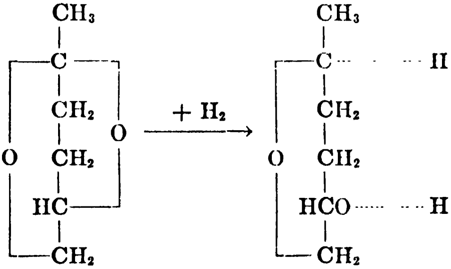
Reduction of 5,6-Dihydroxyhexanone-2

In conclusion, an interesting reaction of the 5,6-dihydroxyhexanone-2 may be mentioned which in a way, may go to show the readiness with which the substance undergoes desaturation. When the dihydroxyketone in acetic acid solution is acted upon by hydrogen gas in the presence of Adams' platinum dioxide catalyst, the molecule loses 1 hydroxyl and the resulting substance forms on acetylation only a monoacetyl derivative. The most probable structure of the new substance is that of a tetrahydrofuran or of a tetrahydropyran derivative (XI or XII).


XI


XII

The formation of this substance from the original cycloketone may take place in three different ways. (*a*) It may be preceded by the formation of the anhydride IX or X. (*b*) It may be preceded by the formation of the anhydride VII, which undergoes reductive opening of one ring, 1 hydrogen atom being added to carbon atom 2, and the other to the oxygen atom involved in the ring formation.


XIII XIV

(*c*) The hydroxyl in position (2) of the cyclic ketone may undergo direct reduction. The last possibility does not seem to us probable in view of the fact that experiments in our laboratory have shown that tertiary aliphatic carbinols do not undergo reduction on treatment with hydrogen in the presence of Adams' catalyst. Of the other two reactions, the first one seems to us the more probable.

EXPERIMENTAL

5,6-Dihydroxyhexanone-2

5,6-Dihydroxyhexanone-2 was prepared from α-acetyl-δ-chloro-γ-valerolactone by the procedure of Traube and Lehmann[3] modified slightly, as follows: To the cooled solution of 47 gm. of sodium in 800 cc. of absolute alcohol 260 gm. of freshly distilled ethylacetoacetate were slowly added. To this cooled solution (temperature 25–30°) in turn were added 190 gm. of redistilled epichlorohydrin dissolved in 250 cc. of absolute alcohol in such a manner that the temperature did not rise above 35°. If the temperature was kept at zero degrees, a poor yield of acetylchlorolactone was obtained. The reaction mixture was continuously stirred. After allowing the mixture to stand overnight, it was concentrated under reduced pressure and the residue was treated with 2000 cc. of 5 per cent sulfuric acid in a separatory funnel. An oil separated which was dissolved in ether and dried over sodium sulfate. After removal of the solvent the lactone distilled at 110–112° at 0.2 mm. Yield 230 gm. (65 per cent).

Redistilled α-acetyl-δ-chloro-γ-valerolactone (214 gm.) was refluxed with a solution of 85 gm. (0.5 mol) of potassium carbonate in 1615 cc. of water for 4 hours. The oil gradually dissolved with evolution of carbon dioxide. The solution was concentrated at a low temperature under diminished pressure. The residue was dissolved in absolute alcohol, the solution filtered with suction from the potassium chloride, and then concentrated under reduced pressure. In order to remove as much as possible of the inorganic material, the residue was repeatedly dissolved in absolute alcohol and to the solution a little dry ether was added. The solution then was distilled under reduced pressure from a short 45 cc. Claisen distilling flask; the 5,6-dihydroxyhexanone-2 distilled at 120° at 0.3 mm. The total yield was 112 gm. or 70 per cent calculated on the basis of the chlorolactone used. The redistilled substance analyzed as follows:

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[3] Traube, W., and Lehmann, E., *Ber. chem. Ges.*, **34**, 1971 (1901).

9.285 mg. substance: 18.645 mg. CO_2 and 7.505 mg. H_2O.
$C_6H_{12}O_3$ (132.13). Calculated. C 54.51, H 9.16.
Found. " 54.75, " 9.04.

Molecular Weight Determination by Cryoscopy.—14.196 gm. of water; 0.3676 gm. of substance; 0.371° difference in freezing temperatures.

$$\text{Mol. wt.} = 18.5 \times \frac{100 \times 0.3676}{14.196 \times 0.371} = 129. \quad \text{Mol. wt. calculated, } 132.13.$$

Molecular Refraction Determination.—

$n_{25} = 1.4673$; $d_{25} = 1.118$; $[M]_D = 32.81$.
$[M]_D$ calculated for straight chain, 32.967.
$[M]_D$ " " cyclic form, 32.40.
Average, 32.68.

Diacetyl-5,6-Dihydroxyhexanone-2 ($CH_3COCH_2CH_2CH(OCOCH_3)$-$CH_2OCOCH_3$)

6 gm. of dihydroxyhexanone (b.p. 132° at 2 mm.) were dissolved in 16 cc. of acetic anhydride to which 16 cc. of dry pyridine had been added. The mixture was allowed to stand overnight. After removal of the solvents the diacetate derivative distilled at 112–114° at 0.6 mm. (8.7 gm.). It was insoluble in water. It analyzed as follows:

7.025 mg. substance: 14.410 mg. CO_2 and 4.850 mg. H_2O.
$C_{10}H_{16}O_5$ (216.18). Calculated. C 55.53, H 7.46.
Found. " 55.93, " 7.72.

Acetyl Number.—To 0.1373 gm. of substance were added 35.0 cc. of 0.1 N NaOH. After allowing the mixture to stand overnight 22.3 cc. of 0.1 N HCl were required for neutralization. Calculated, 519; found, 519.

Molecular Refraction.—The value points to an open chain formula.

$n_{25} = 1.4398$; $d_{25} = 1.0952$; $[M]_D = 52.00$.
$[M]_D$ calculated for open chain, 51.696.
$[M]_D$ " " cyclo form, 51.128.

Semicarbazone of Diacetyldihydroxyhexanone.—Two structures are theoretically possible for the diacetate, one a cyclic and the other a keto form. The existence of the latter at least in predominating quantity is seen from the formation of the semicarbazone in a good yield. The semicarbazone was prepared in the following way. 0.8 gm. of potassium acetate and 0.8 gm. of semicarbazide hydrochloride were dissolved in 2.3 cc. of water. To the solution was added 1 gm. of the diacetate and about 1 cc. of methyl alcohol. The reaction took place with evolution of heat. On standing for 1½ days approximately 1.3 gm. of the crystalline semicarbazone were obtained. On repeated recrystallization from methyl alcohol the substance melted at 112° (with decomposition). It analyzed as follows:

0.5065 mg. substance: 8.975 mg. CO_2 and 3.210 mg. H_2O.
0.4530 " " : 0.635 cc. N (38°, 752 mm.).

$C_{11}H_{19}O_6N_3$ (273.24).	Calculated.	C 48.33, H 7.01, N 15.38.
	Found.	" 48.32, " 7.09, " 15.62.

Methylcyclo-5,6-Dihydroxyhexanone-2 (*III or IV*)

This substance is readily obtained by dissolving the dihydroxyhexanone in 10 times its weight of dry methyl alcohol containing 0.5 per cent of hydrogen chloride. After 4 hours, the hydrogen chloride was removed with dry silver carbonate. The filtrate was concentrated and the residue distilled at 66–69° at 1.5 mm. In this manner from 10 gm. of dihydroxyhexanone 10 gm. (or 90 per cent) of the methylcyclo derivative were obtained. The analysis was as follows:

5.525 mg. substance: 11.650 mg. CO_2 and 4.615 mg. H_2O.
0.1752 gm. " : 0.2742 gm. AgI (Zeisel).

$C_7H_{14}O_3$ (146.15).	Calculated.	C 57.50, H 9.66, OCH_3 21.21.
	Found.	" 57.50, " 9.34, " 20.66.

The value of molecular refraction was as follows:

$n_{25} = 1.4496$; $d_{25} = 1.0614$; $[M]_D$ calculated 37.13; found 36.97.

Acetyl Methylcyclo-5,6-Dihydroxyhexanone-2.—3 gm. of the methylcyclodihydroxyhexanone were dissolved in a mixture of 9 cc. of acetic anhydride and 9 cc. of dry pyridine. On distillation a fraction was obtained which distilled at 53–54° at 0.1 to 0.2 mm.

Yield 3.3 gm. This material was redistilled and a fraction was collected which boiled at 102–104° at 16 mm. It analyzed as follows:

4.985 mg. substance: 10.580 mg. CO_2 and 3.835 mg. H_2O.
$C_9H_{16}O_4$ (188.17). Calculated. C 57.42, H 8.57.
Found. " 57.87, " 8.60.

Acetyl Number.—0.1666 gm. of substance and 40.0 cc. of 0.1 N NaOH were allowed to stand overnight. 30.4 cc. of 0.1 N HCl were required for neutralization. Calculated, 298; found, 323.

Dimethylcyclodihydroxyhexanone

18 gm. of methylcyclo-5,6-dihydroxyhexanone-2 were methylated by means of silver oxide and methyl iodide. A total quantity of 50 gm. of silver oxide and 150 gm. of methyl iodide were used and added in four portions. Heating of this reaction mixture was continued for 5 hours. The mixture was then cooled and extracted with dry ether and the ethereal solution dried with anhydrous sodium sulfate. The solvent was removed and three fractions were obtained on distillation.

Fraction I, boiling at 74° at 17 mm., weight 9.4 gm.
" II, " " 75–82° at 17 mm.
" III, viscous residue; weight 1.2 gm.

Fraction I.—It had the composition of dimethylcyclodihydroxyhexanone.

5.250 mg. substance: 11.525 mg. CO_2 and 4.895 mg. H_2O.
0.1076 gm. " : 0.3118 gm. AgI (Zeisel).
$C_8H_{16}O_3$ (160.17). Calculated. C 59.96, H 10.07, OCH_3 38.75.
Found. " 59.85, " 10.43, " 38.25.

The value of the molecular refraction corresponded to the cyclo form.

n_{25} = 1.4302; d_{25} = 0.9922. $[M]_D^{25}$ calculated for the cyclo form 41.87; found 41.72.

Fraction III. Condensation Product.—This fraction represented 1.2 gm. of a viscous residue. It had the composition of an anhydrodihydroxyhexanone.

3.450 mg. substance: 8.005 mg. CO_2 and 2.750 mg. H_2O.
$C_6H_{10}O_2$ (114.11). Calculated. C 63.12, H 8.83.
Found. " 63.27, " 8.91.

The substance was acetylated by means of acetic anhydride and pyridine. After removal of the acetylation mixture, attempts at fractional distillation under the pressure of about 1 mm. were unsuccessful although the bath was heated to 190°. The substance had the composition of a monoacetylanhydrodihydroxyhexanone.

5.005 mg. substance: 11.245 mg. CO_2 and 3.625 mg. H_2O.

$C_8H_{12}O_3$ (156.14).	Calculated.	C 61.51, H 7.75.
	Found.	" 61.26, " 8.10.

Acetyl Number.—0.1960 gm. of substance was refluxed for 1½ hours with 40.0 cc. of 0.1 N NaOH. 29.0 cc. of 0.1 N HCl were required for neutralization. Calculated, 359; found, 308.

In another methylation experiment 5 gm. of methylcyclodihydroxyketone with 45 gm. of silver oxide and an excess of methyl iodide were refluxed for about 20 hours.

A small amount of a substance was obtained which boiled at 60° at about 17 mm. According to its analysis, it represents monomethoxydihydroxyhexanone anhydride.

5.030 mg. substance: 11.945 mg. CO_2 and 4.250 mg. H_2O.
0.1806 gm. " : 0.3312 gm. AgI (Zeisel).

$C_7H_{12}O_2$ (128.13).	Calculated.	C 65.58,	H 9.44,	OCH_3 24.19.
	Found.	" 64.75,	" 9.45,	" 24.20.

Condensation of 5,6-Dihydroxyhexanone-2

This reaction takes place even at a low temperature. It is probable that a trace of a substance present in the 5,6-dihydroxyhexanone catalyzes this reaction since the yield of the disaccharide on short standing sometimes was higher than when the substance was allowed to stand for a longer period.

Thus, 36 gm. of redistilled 5,6-dihydroxyhexanone-2 were kept at 25° for 12 days and then subjected to distillation. The first fraction consisted of a small quantity of water after which only 24 gm. of substance distilled at the boiling temperature of the dihydroxyketone. It had been also observed that during the distillation further condensation, to a small extent, had taken place. The residue was distilled at about 1 mm. pressure. A fraction was obtained which distilled at 175–180° at 0.3 to 0.4 mm.

The substance had the composition of a dihydroxyhexanonyl dihydroxyhexanone.

6.355 mg. substance: 13.620 mg. CO_2 and 5.150 mg. H_2O.
$C_{12}H_{22}O_5$ (246.26). Calculated. C 58.50, H 9.01.
Found. " 58.44, " 9.06.

Acetylation of Distillation Residue.—In another experiment 31 gm. of 5,6-dihydroxyhexanone-2 were kept at room temperature. This time only one-half of the amount of dihydroxyhexanone was recovered by distillation. 5 gm. of the residue were dissolved in a mixture of 15 cc. of acetic anhydride and 15 cc. of dry pyridine. The following day the reaction product was fractionated. The fraction boiling from 170–180° at 0.2 to 0.3 mm. had the composition of a diacetyl di-(dihydroxyhexanone) anhydride.

3.700 mg. substance: 7.945 mg. CO_2 and 2.595 mg. H_2O.
$C_{16}H_{26}O_7$ (330.29). Calculated. C 58.15, H 7.94.
Found. " 58.55, " 7.84.

Acetyl Number.—0.1318 gm. of substance was refluxed for 2 hours with 40.0 cc. of 0.1 N NaOH. 31.7 cc. of 0.1 N HCl were required for neutralization. Calculated, 340; found, 353.

In another experiment freshly distilled dihydroxyketone was allowed to stand at about 0° for 14 days. On being distilled, only 84 per cent of the original substance was recovered. In order to prevent condensation on standing it is therefore advisable to preserve the dihydroxyketone in a solvent.

Methylcyclodihydroxyhexanone from Di-(Dihydroxyhexanone) Anhydride

1.7 gm. of the disaccharide were dissolved in 12.5 cc. of methyl alcohol containing 0.5 per cent of hydrogen chloride. After allowing the mixture to stand at room temperature it was neutralized with silver carbonate. The filtered solution was concentrated at a low temperature. At the temperature of 101–103° (15 mm.) a fraction of 1.2 gm. was obtained which had the composition of methylcyclodihydroxyhexanone.

3.290 mg. substance: 6.990 mg. CO_2 and 2.980 mg. H_2O.
0.0964 gm. " : 0.1554 gm. AgI (Zeisel).
$C_7H_{14}O_3$ (146.15). Calculated. C 57.50, H 9.66, OCH_3 21.21.
Found. " 57.93, " 10.12, " 21.26.

Polymerization Product Obtained from 5,6-Dihydroxyhexanone-2 with Concentrated Sulfuric Acid. (According to Hibbert and Timm[2]*)*

To 9 gm. of 5,6-dihydroxyhexanone-2 was added 0.009 gm. of concentrated sulfuric acid. The mixture was stirred and heated at 155° for 4 minutes. The dark, resinous material was then kept at 100° under slightly reduced pressure. The substance had the composition of an anhydride of 5,6-dihydroxyhexanone-2.

4.760 mg. substance: 11.075 mg. CO_2 and 3.840 mg. H_2O.

$(C_6H_{10}O_2)_x$ (114.11).	Calculated.	C 63.12, H 8.83.
	Found.	" 63.45, " 9.02.

Acetylation of Polymeric Anhydride of Dihydroxyhexanone.—2.1 gm. of the above polymeric anhydride of the dihydroxyhexanone were dissolved in 4.5 cc. of dry pyridine. To this solution 4 cc. of acetic anhydride were added, some heat being evolved. After 36 hours standing the mixture was taken up in chloroform, water was added, and the chloroform solution was washed with dilute solutions of sulfuric acid and sodium carbonate. After drying of the washed chloroform solution with sodium sulfate, the solvent was evaporated.

An attempt was made to distil the residue under reduced pressure but no distillate was obtained. The new compound had the composition of a monoacetate of a polymer dihydroxyhexanone anhydride.

3.600 mg. substance: 8.125 mg. CO_2 and 2.410 mg. H_2O.

$(C_8H_{12}O_3)_x$ (156.14).	Calculated.	C 61.51, H 7.75.
	Found.	" 61.54, " 7.49.

Acetyl Number.—0.2031 gm. of substance required 13.7 cc. of 0.1 N NaOH for neutralization. Calculated, 359; found, 378.

Anhydride of 5,6-Dihydroxyhexanone-2

40 gm. of 5,6-dihydroxyhexanone-2 were heated in a double neck distilling flask at a temperature of 150° for 9 hours. The heating was carried out for 2 to 3 hours at a time, the material being kept in the ice box in between times, about 20 hours. The reaction mixture was then distilled under a pressure of less than 1 mm. The receiving flask was water-cooled, but the vapor leading to the

vacuum pump was passed through a U-tube which was cooled to a very low temperature with solid CO_2 and alcohol. On gradually raising the bath temperature, a crystalline material was soon observed in the cold U-tube. It consisted of a mixture of water and anhydride. The temperature of the bath was finally raised to about 160° when most of the material had distilled over. In the first, the water-cooled receiver, a mixture of substances was obtained. The crystalline material in the carbon dioxide-cooled flask was immediately dissolved in dry ether and separated from a small aqueous layer which was formed occasionally. The ether solution was dried with sodium sulfate and kept overnight at 0°. After removal of the ether from the filtered solution the remaining mobile liquid was fractionated. In some instances the non-fractionated material was used. The substance had the composition of an anhydride of 5,6-dihydroxyhexanone-2. On fractionation a portion was obtained which boiled at 40° at 20 mm. or at 55° at 42 mm. This substance had the following composition.

3.860 mg. substance: 8.980 mg. CO_2 and 2.990 mg. H_2O.
$C_6H_{10}O_2$ (114.11). Calculated. C 63.12, H 8.83.
Found. " 63.44, " 8.55.

Molecular Weight Determination by Cryoscopy.—This determination was carried out on a non-fractionated material. 8.093 gm. of benzene; 0.1025 gm. of substance; 0.532° difference of freezing temperatures. Mol. wt. of the monomolecular form ($C_6H_{10}O_2$) calculated, 114.1; found, 121.7.

Molecular Refraction.—The molecular refraction as well as the parachor were determined on a pure freshly distilled sample of 5,6-dihydroxycyclohexanone anhydride. The values obtained from determination of the molecular refraction agree with that calculated for a molecule containing two oxygen bridges.

$n_{25} = 1.4350$; $d_{25} = 1.0423$; $[M]_D = 28.57$.
$[M]_D$ calculated (formula VII) 28.79, (formula IX or X) 30.41.

Determination of Parachor.—The values found for the distilled anhydride are also in better agreement with a molecule containing two oxygen bridges (formula VII) than with a molecule containing a double bond and one oxygen bridge (formulas IX or X).

The parachor was determined[4] according to Sugden.[5] The calculations were made using his simplified formula:

$$\text{Parachor} = \frac{M}{D}\gamma^{\frac{1}{4}}$$

where M is molecular weight, D density, and γ surface tension. The constant of the surface tension apparatus was established by determining the surface tension of known substances. The constant of the apparatus was 0.00380_4. The parachor of ether, chloroform, and carbon tetrachloride were 209.5, 183.2, and 220 respectively. Calculated 210.2, 184.8, and 222.0 respectively.

Difference of pressure observed on water gauge = 8245 dynes per cm.^{2}
$\gamma_{26} = 31.58$. Parachor found = 259.2.
Calculated, formula VII 254.4; formula IX 271.5; formula X 269.1.

Occasionally, the ethereal extract of the distillate in the carbon dioxide-alcohol-cooled tube turned viscous so rapidly on evaporation of the ether that no appreciable amount of the monomolecular anhydride could be obtained.

Acetate of Anhydride of 5,6-Dihydroxyhexanone-2

3 gm. of non-fractionated anhydride of 5,6-dihydroxyhexanone-2 were dissolved in a mixture of 7 cc. of pyridine and 6 cc. of acetic anhydride. After standing for 20 hours the mixture was concentrated under reduced pressure. The residue was taken up in chloroform and washed with dilute solutions of sulfuric acid, sodium carbonate, and finally with water. The dried chloroform solution was concentrated and 2 gm. of a substance boiling at 58° at 2 mm. pressure were obtained. The substance had the composition of a monoacetate of an anhydride of dihydroxyhexanone.

5.330 mg. substance: 12.000 mg. CO_2 and 3.900 mg. H_2O.
$C_8H_{12}O_3$ (156.14). Calculated. C 61.51, H 7.75.
Found. " 61.39, " 8.19.

Acetyl Number.—0.0925 gm. of substance required 5.8 cc. of 0.1 N NaOH for neutralization. Calculated, 359; found, 352.

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[4] All the parachor determinations reported in this paper were carried out by Dr. Alexandre Rothen.

[5] Sugden, S., *J. Chem. Soc.*, **125**, 1177 (1924).

The values of the molecular refraction and of the parachor of the substance were in agreement with those calculated for the acetate of anhydrodihydroxyhexanone (acetate of substance IX or X).

Molecular Refraction.
n_{25} = 1.4490; d_4^{25} = 1.0575; calculated $[M]_D$ 39.77; found $[M]_D$ 39.55.
Parachor.—Difference of pressure observed on water gauge = 8490 dynes per cm^2.
γ_{25} (surface tension) = 32.50; d_4^{25} = 1.0575.
Parachor found = 352.6; parachor calculated = 352.9 (6-membered ring).
" " = 355.3 (5- " ").

Hydrogenation of Acetate of Anhydride of 5,6-Dihydroxyhexanone-2

0.05 gm. of platinum dioxide (Adams' catalyst) and 4 cc. of 80 per cent alcohol were placed in a small hydrogenation vessel. After reduction of the catalyst, 1 gm. of the above acetyl derivative dissolved in 7 cc. of alcohol was added. A rapid absorption of 135 cc. of hydrogen took place. Further shaking did not increase the absorption of hydrogen. The filtrate from the catalyst was freed from its solvent by distillation, the residue was transferred into a 1.5 cc. double neck distilling flask. 0.5 cc. of a substance distilled at 95° (12 mm.). It had the composition of a dihydro derivative of the acetylated anhydride.

4.065 mg. substance: 9.050 mg. CO_2 and 3.300 mg. H_2O.
$C_8H_{14}O_3$ (158.15). Calculated. C 60.73, H 8.92.
Found. " 60.71, " 9.08.

Acetyl Number.—0.1009 gm. of substance required 6.3 cc. of 0.1 N NaOH for neutralization. Calculated, 355; found, 350.

Products Obtained in the Water-Cooled Receiver

In the water-cooled receiving flask (see anhydride of 5,6-dihydroxyhexanone-2) two substances were identified after acetylation, namely dihydroxyhexanone anhydride of type IX or X and 5,6-dihydroxyhexanone-2. The material separated at times into two layers. The top layer was decanted and acetylated with pyridine and acetic anhydride. It yielded an anhydrodihydroxyhexanone acetate boiling at 50–53° at 1 mm.

5.246 mg. substance: 11.740 mg. CO_2 and 3.615 mg. H_2O.
$C_8H_{12}O_3$ (156.14). Calculated. C 61.51, H 7.75.
Found. " 61.03, " 7.71.

Acetyl Number.—0.0910 gm. of substance required 6.2 cc. of 0.1 N NaOH for neutralization. Calculated, 359; found, 382.

There was a certain amount of a higher boiling fraction which distilled over a wide temperature range.

The bottom layer was also acetylated with pyridine and acetic anhydride. The main fraction distilled at 105° and 0.8 mm. It had the composition of dihydroxyhexanonediacetate.

4.550 mg. substance: 9.420 mg. CO_2 and 3.130 mg. H_2O.
$C_{10}H_{16}O_5$ (216.18). Calculated. C 55.53, H 7.46.
Found. " 56.45, " 7.69.

Acetyl Number.—0.1983 gm. of substance required 16.8 cc. of 0.1 N NaOH for neutralization. Calculated, 519; found, 475.

Polymerized Anhydro-5,6-Dihydroxyhexanone-2

20 gm. of 5,6-dihydroxyhexanone-2 were heated in a manner similar to that previously described (three times for 3½ hours at approximately 150°). On distillation of this substance at the bath temperature of 230° under reduced pressure only water came over. The residue was a hard brown resin and had a composition approximating that of a polymerized 5,6-dihydroxyhexanone anhydride.

4.440 mg. substance: 10.445 mg. CO_2 and 3.565 mg. H_2O.
$(C_6H_{10}O_2)_x$ (114.11). Calculated. C 63.12, H 8.83.
Found. " 64.15, " 8.98.

Acetate of Polymerized 5,6-Dihydroxyhexanone Anhydride.—5 gm. of the polymerized anhydride just described were acetylated with a mixture of 11 cc. of dry pyridine and 8 cc. of acetic anhydride. The following day the mixture was taken up in 100 cc. of chloroform, washed with dilute solutions of sulfuric acid and sodium carbonate and finally with water. On evaporation of the solvent and distillation of the residue a very small fraction was obtained which had the character of the anhydride of the 5,6-dihydroxyhexanone-2. The main portion could not be distilled even under greatly reduced pressure. It had the approximate composition of the acetate of the anhydrodihydroxyhexanone.

$(C_8H_{12}O_3)_x$ (156.14). Calculated. C 61.51, H 7.75.
Found. " 62.05, " 7.48.

Acetyl Number.—0.1554 gm. of substance required 11.1 cc. of 0.1 N NaOH for neutralization. Calculated, 359; found, 400.

Hydrogenation of Acetate of Polymerized Dihydroxyhexanone Anhydride.—0.05 gm. of Adams' platinum dioxide catalyst was reduced in an alcoholic medium and 1 gm. of the above polymerized anhydride acetate was then added. Within 4 hours 20 cc. of hydrogen were absorbed. Further shaking did not increase the absorption although the theory required 141 cc.

Treatment of Polymerized 5,6-Dihydroxyhexanone Anhydride with Methyl Alcohol Containing Hydrogen Chloride

If the polymerized anhydride was treated with cold methyl alcohol containing 0.5 per cent of hydrogen chloride, the material remained practically unchanged. If, on the other hand, it was refluxed with the same amount of the solvent, a semisolid substance was obtained which contained methoxyl groups. Thus, 5 gm. of polymerized anhydride were refluxed with 50 cc. of methyl alcohol containing 0.5 per cent of hydrogen chloride for 4 hours. The following morning the hydrogen chloride was removed with silver carbonate and the methyl alcohol evaporated under reduced pressure. Nothing distilled at the bath temperature of 170° the pressure being less than 1 mm. The composition of the substance was approximately that of a compound consisting of five dihydroxyhexanone rests containing one methyl group.

5.320 mg. substance: 12.090 mg. CO_2 and 4.190 mg. H_2O.
14.485 " " : 5.845 " AgI (Zeisel).
$C_{31}H_{54}O_{11}$. Calculated. C 61.76, H 9.03, OCH_3 5.15.
Found. " 61.97, " 8.81, " 5.32.

Distilled Polymeric Anhydride of Dihydroxyhexanone

If the solid polymeric anhydride was heated in a bath at a temperature of 230–290° and under highly reduced pressure, a small amount of a yellow viscous material distilled at 130–180° at 0.5 mm. It had the composition of a dihydroxyhexanone anhydride.

3.815 mg. substance: 8.780 mg. CO_2 and 3.055 mg. H_2O.
$(C_6H_{10}O_2)_x$ (114.11). Calculated. C 63.12, H 8.83.
Found. " 62.76, " 8.96.

Acetylation of Distilled Polymeric Anhydride of Dihydroxyhexanone.—1.5 gm. of the above substance were acetylated with a mixture of 3 cc. of pyridine and 3 cc. of acetic anhydride. After standing for 15 hours, the mixture was taken up in chloroform, washed with dilute solutions of sulfuric acid and sodium carbonate and finally with water. After drying the solvent was removed by distillation; the residue distilled approximately at the temperature of the boiling point of the acetate of the monomeric dihydroxyhexanone anhydride. It was not analyzed. A second fraction was obtained which boiled at 92–125° at 0.8 mm. It had the following composition: C 60.6; H 8.31; acetyl number 267. The remaining 0.7 gm. of light brown viscous material had practically the composition of an acetate of dihydroxyhexanone anhydride.

3.610 mg. substance, 8.090 mg. CO_2 and 2.610 mg. H_2O.
$(C_8H_{12}O_3)_x$ (156.14). Calculated. C 61.51, H 7.75.
Found. " 61.11, " 8.08.

Acetyl Number.—0.1099 gm. of substance required 6.2 cc. of 0.1 N NaOH for neutralization. Calculated, 359; found, 319.

The brown distillation residue obtained from the fraction boiling at 130–180° at 0.5 mm. possessed the composition of C 65.55, and H 9.06.

Hydrogenation of 5,6-Dihydroxyhexanone-2

7 gm. of freshly distilled 5,6-dihydroxyhexanone-2 were dissolved in 40 cc. of glacial acetic acid. To this solution was added 0.12 gm. of Adams' platinum dioxide catalyst. After elimination of the air in the flask, a pressure of 1.5 atmospheres of hydrogen was applied. In 4 hours approximately 1800 cc. of hydrogen were absorbed. After filtering off the catalyst most of the acetic acid was evaporated under diminished pressure. The residue was taken up in ether and the mixture was washed with a dilute solution of sodium carbonate. The dried ether solution then was fractionated and 3 gm. of a fraction boiling at 70–73° at 14 mm. were obtained. The substance did not reduce Fehling's solution nor did it form a semicarbazone. It had the composition of a monohydroxyhexanone and has the structure of a tetrahydrofuran or tetrahydropyran derivative (formula XI or XII). The analysis was as follows:

3.775 mg. substance: 8.600 mg. CO_2 and 3.495 mg. H_2O.
$C_6H_{12}O_2$ (116.13). Calculated. C 62.02, H 10.42.
Found. " 62.12, " 10.36.

Acetate of Monohydroxy Derivative.—To 2.6 gm. of the above substance was addded a mixture of 5 cc. of pyridine and 5 cc. of acetic anhydride. The mixture was allowed to stand for 5 hours and was then concentrated under reduced pressure. The residue was taken up in chloroform and the solution was washed with dilute sulfuric acid and sodium carbonate. The dried chloroform solution gave on fractionation 2 cc. of a substance which boiled at 88° at 13 mm. The substance possessed the composition of a monoacetyl derivative.

5.350 mg. substance: 11.955 mg. CO_2 and 4.235 mg. H_2O.
$C_8H_{14}O_3$ (158.15). Calculated. C 60.73, H 8.92.
Found. " 60.93, " 8.85.

Acetyl Number.—0.1072 gm. of substance required 6.8 cc. of 0.1 N NaOH for neutralization. Calculated, 355; found, 356.

Molecular Refraction.—The value of the molecular refraction of the substance was in agreement with its cyclic structure.

$d^{25} = 1.0175$; $n^{25} = 1.4321$; $[M]_D = 40.32$.
$[M]_D$ calculated 40.24 (cyclic form).
$[M]_D$ " 40.81 (open chain).

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THE PARTIAL DEHYDROGENATION OF α- AND β-AMYRIN

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In recent years polyterpene chemistry has been greatly advanced, especially through the work of Ruzicka and his coworkers, by the application of the method of exhaustive dehydrogenation with sulfur, and more recently by the use of the selenium method of Diels.[1] During the past year Ruzicka has extended the study to the more complex triterpene derivatives, such as the amyrins[2] and various sapogenins.[3] Although frequent reference has been made to the probable close relationship of the sapogenins, sterins, and other higher terpenes, it is very necessary to continue the study in order to determine the real particulars of these relationships. In a report of their experiments on the amyrins Ruzicka, Huyser, Pfeiffer, and Seidel[2] obtained by dehydrogenation with sulfur[4] and preferably with selenium a hydrocarbon, $C_{13}H_{14}$, which was isolated as the picrate or styphnate and was found to be a trimethylnaphthalene. This hydrocarbon on oxidation gave a naphthalenetricarbonic acid. Since in a subsequent work Ruzicka and van Veen[3] succeeded in obtaining the same hydrocarbon by dehydrogenation with selenium of a series of sapogenins and sterins, although with certain exceptions, they have proposed the name sapotalin for this trimethylnaphthalene. Although the mechanism of the formation of this substance is still unknown, they have been able to conclude that there is a close relationship between a number of these sapogenins and natural triterpene derivatives. This conclusion in so far as β-amyrin and hederagenin are concerned has been extended by our own work which is presented in the present and following communications.

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[1] Diels, O., and Karstens, A., *Ber. chem. Ges.*, **60**, 2323 (1927). Diels, O., Gädke, W., and Körding, P., *Ann. Chem.*, **459**, 1 (1927).

[2] Ruzicka, L., Huyser, H. W., Pfeiffer, M., and Seidel, C. F., *Ann. Chem.*, **471**, 21 (1929).

[3] Ruzicka, L., and van Veen, A. G., *Z. physiol. Chem.*, **184**, 69 (1929); *Rec. trav. chim. Pays-Bas*, **48**, 1018 (1929).

[4] Brunner, O., (*Monatsh. Chem.*, **50**, 287 (1928)) has also recently reported experiments on the dehydrogenation of the mixed amyrins with sulfur which, however, were very inconclusive.

Before the appearance of the experiments of Ruzicka and coworkers on the dehydrogenation of amyrin, we had already in progress a study of the dehydrogenation of this substance. Since our point of view and immediate objective differed from those of these workers we have therefore continued these studies. In the dehydrogenation experiments already mentioned, the purpose has been exhaustive treatment in order to achieve complete conversion into aromatic hydrocarbons which might be easier of recognition. It occurred to us that it might be possible to interrupt the dehydrogenation at proper points and so obtain substances which represent partial dehydrogenation before too deep seated changes have occurred. The study of such derivatives might then prove useful in giving clues to the structure of the parent substances. With this in view, the examination of a series of substances has been begun, including the sapogenins, resin alcohols, and other polyterpene derivatives as well as the cardiac aglucones and other members of the saturated polycyclic group. Our preliminary results have demonstrated the possible usefulness of this method of approach. We shall present here the experience with the amyrins.

These experiments have been made with pure α- and β-amyrin which were prepared by the method of Vesterberg and Westerlind[5] from the crude mixture of the isomers obtained from gum elemi as well as with their benzoates. When α- and β-amyrin were heated in an atmosphere of nitrogen with an equal weight of sulfur, a slow but steady evolution of hydrogen sulfide occurred at 200–210°, which continued for several hours with the bath at this temperature, and then gradually ceased. In the case of the benzoates it was necessary to go somewhat higher, to 220–230°, in order to obtain a parallel result. If in any case the temperature was raised, as previous observations have already shown, there was of course further dehydrogenation with extensive carbonization. If, however, the reaction was interrupted at this point, it was possible to isolate definite transformation products. This was accomplished by extraction of the reaction mixture and fractionation of the soluble material at low pressure. The resulting resins were then crystallized from appropriate solvents. But the substances obtained in the case of β-amyrin and its benzoate differed in character from those obtained from the α compounds. β-amyrin or its benzoate yielded sulfur compounds which from the formulations deduced from the analytical results, respectively $C_{30}H_{44}OS$ and $C_{37}H_{48}O_2S$, have been formed by the removal of 3 mols of hydrogen and the introduction of 1 sulfur atom. The reactions occurring in the case of β-amyrin and its benzoate were identical since the thio compounds obtained from both sources were interconvertible by benzoylation or saponification. The yield in the case of the benzoate (about 50 per cent) was somewhat better than in the case of β-amyrin itself (35 per cent). The secondary hydroxyl group of β-amyrin is, therefore, retained in the sulfur derivative.

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[5] Vesterberg, K. A., and Westerlind, S., *Ann. Chem.*, **428**, 243 (1922).

The sulfur of the thio compounds is apparently of complex thiophene character since it cannot be removed as hydrogen sulfide on heating with strong alcoholic alkali. On the other hand, it was readily removed in oxidation experiments, as given below. Attempts to demonstrate the presence of new double bonds by catalytic hydrogenation experiments were unsuccessful. This fact, however, does not exclude the presence of double bonds.

In the oxidation experiments the benzoate of the thio compound was employed in order to avoid complications which might arise by participation in the reaction of the secondary hydroxyl group. With permanganate in acetic acid, it was gradually oxidized with the formation of at least two substances of neutral character which no longer contained sulfur. Although it was possible to separate these substances by fractional recrystallization, it was found preferable to saponify the crude benzoate mixture. From the resulting mixture of hydroxy compounds the two substances were separated by fractional recrystallization and gave analytical figures which agreed best with the formulæ $C_{30}H_{46}O_3$ and $C_{30}H_{44}O_4$ respectively.

On acylation both of these substances gave only a monoacetate or a monobenzoate, the latter proving to be identical in each case with the benzoates obtained directly in the above oxidation. Therefore, aside from the secondary hydroxyl group originally present in amyrin, no other hydroxyl group can be detected by acylation. The substance $C_{30}H_{46}O_3$ proved to be a ketone since it yielded, although with some difficulty, a monooxime. By the Clemmensen method only 1 oxygen atom could be replaced by hydrogen. The remaining oxygen atom introduced during the oxidation is, therefore, of undetermined character although it is presumably a hydroxyl. The substance resisted all attempts to cleave it on heating with strong alkali.

The substance $C_{30}H_{44}O_4$ is apparently a lactone. Although it resisted boiling in 5 per cent alcoholic alkali for hours, when heated with 10 per cent alkali at 130° it was converted into a monobasic acid, $C_{30}H_{46}O_5$. The same substance was encountered in the reaction mixture obtained on saponification of the crude benzoate mixture. The lactone did not react with hydroxylamine. It formed only a monoacetate and a monobenzoate and the latter proved to be identical with that isolated directly from the oxidation mixture. No indication of the absorption of hydrogen was obtained in experiments on catalytic hydrogenation.

The above ketone, however, was found to absorb catalytically activated hydrogen and the resulting substance appeared to be a mixture of isomers. The analytical figures, although not in close agreement, approximated those for amyrin, indicating definitely the loss of the 2 extra oxygen atoms during the hydrogenation, and since it still gave a benzoate, the retention of the original secondary hydroxyl.

Owing to the temporary lack of sufficient material we have been forced to interrupt this work for the present. With the data at hand it is perhaps premature to suggest a picture of the mechanism of the formation of these oxidation products from the thio compound and much less the relationship of the latter to the parent triterpene alcohol. Although several interpretations are possible, we shall leave this rather until further work has been done.

In the recent work of Ruzicka with Pfeiffer[6] and with van Veen[7] on the dehydrogenation of elemol with sulfur, a sulfur-containing dehydrogenation product has also been obtained for which a thionaphthalene structure has been proposed. The possibility of any structural analogy between this substance and our own must be left for the present. It might be further noted that in the dehydrogenation of colophonium oil with sulfur along with retene, a crystalline sulfur compound, $C_{18}H_{16}S$, has been described by Schultze[8] as a thioretene which was shown to be a secondary product due to the further action of sulfur on retene.

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[6] Ruzicka, L., and Pfeiffer, M., *Helv. Chim. Acta*, **9**, 841 (1926).

[7] Ruzicka, L., and van Veen, A. G., *Ann. Chem.*, **476**, 70 (1929).

In the case of both α-amyrin and its benzoate the reaction with sulfur was found to take a different course, at least in so far as was indicated by the study of the reaction product which was obtained. The reaction with sulfur was performed exactly as in the case of the β-isomers. Much more carbonization occurred than in the case of the β-isomer and a much smaller yield, only about 10 per cent, of a crystalline substance was isolated, which was found to contain no sulfur. The analysis gave figures which agreed with the formulæ $C_{30}H_{48}O$ and $C_{37}H_{52}O_2$ for the free hydroxy compound and the benzoate respectively. This indicates that its formation from α-amyrin has resulted from the loss of 1 mol of hydrogen. Characteristic of dehydro-α-amyrin is its high rotation, $[\alpha] = +358°$, and the indigo blue color which it instantly gives with the Liebermann cholesterol test. For further characterization dehydro-α-amyrin was oxidized with chromic acid to the ketone, dehydro-α-amyrone, from which an oxime was prepared. For the time being, its behavior on oxidation with permanganate has not been studied.

The difference in the behaviors of α- and β-amyrin on partial dehydrogenation with sulfur brings up for the first time the question whether the difference in structure between these isomers is merely stereochemical. On the other hand, as will be seen in the following communication on a derivative of hederagenin, there is a striking parallelism in the behaviors of this sapogenin derivative and β-amyrin, which increases the probability of the close relationship between these substances, since analogous structural features are probably involved in each case in the dehydrogenation and cyclization with sulfur.

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[8] Schultze, W., *Ann. Chem.*, **359**, 129 (1908).

EXPERIMENTAL

Partial Dehydrogenation of β-Amyrin and Benzoate

In the dehydrogenation experiments it was found useful in all cases to employ a long necked 50 cc. Pyrex flask fitted with a ground glass stopper of wash bottle type containing an inlet and an exit tube. The latter was connected with a small trap which was in turn connected with a bubble counter containing about 1 cc. of water. After introduction of the mixture of substance and sulfur into the flask, the stopper joint was made gas-tight with sulfur and air was displaced from the apparatus by a stream of nitrogen. This stream was then discontinued and the flask was heated in a metal bath. The temperature was gradually raised until a slow, steady stream of H_2S was evolved. The temperature was then maintained within 10° of this point as long as appreciable evolution of gas continued. The temperature and duration will be recorded in each case.

Dehydrogenation of β-Amyrin Benzoate.—A mixture of 9.0 gm. of β-amyrin benzoate and an equal amount of sulfur was heated for 4 hours at a bath temperature of 220–230°. The reaction mass was extracted with benzene and the excess sulfur removed by filtration. The benzene solution was evaporated to dryness on the steam bath and the resulting solid was distilled at a pressure of 2 mm. in 3 gm. portions from a 10 cc. Claisen distilling flask. Solidification of the distillate in the delivery tube of the distilling flask was prevented by inserting a copper wire through the delivery tube and extending this wire into the side neck of the Claisen flask. The temperature of the metal bath, in which the flask was immersed, was gradually increased until 275° was attained. At this point practically all of the free sulfur had distilled off. A cherry-colored resin distilled when the temperature of the bath was maintained between 300–350°. The resin was dissolved in a small amount of benzene and precipitated with 2 volumes of methyl alcohol. The precipitate was dissolved in benzene and decolorized with norit. When this solution was concentrated, the substance crystallized in the form of prisms which melted at 224–225°. The yield was 50 per cent.

$[\alpha]_D^{25} = +96°$ (c = 1.017 in pyridine).
4.596 mg. substance: 3.610 mg. H_2O, 13.424 mg. CO_2.
4.648 " " : 3.720 " " 13.600 " "
6.654 " " : 2.755 " $BaSO_4$.
$C_{37}H_{48}O_2S$. Calculated. C 79.80, H 8.70, S 5.75.
Found. (*a*) " 79.66, " 8.79.
(*b*) " 79.80, " 8.96.
(*c*) S 5.68

Molecular weight determination according to the method of Rast[9] gave the following results:

29.160 mg. camphor: 3.234 mg. substance, $\Delta = 8.75$. Mol. wt., calculated, 556.4; found, 570.

The Liebermann cholesterol test gave an amber-colored solution which changed into a reddish brown when warmed. Attempts to benzoylate the substance with pyridine and benzoyl chloride resulted in recovery of unchanged material. When this substance was refluxed for 6 hours in an acetic acid solution of lead acetate, no evidence of the cleavage of sulfur was noted and the starting material was recovered unchanged.

Saponification of the Benzoate, $C_{37}H_{48}O_2S$.—A solution of 0.5 gm. of the above sulfur compound in 50 cc. of 10 per cent alcoholic KOH was refluxed for 5 hours. The product was precipitated from the boiling solution by addition of water. When recrystallized from alcohol it separated in the form of fine needles which melted at 200–201°.

$[\alpha]_D^{30} = +88°$ (c = 1.017 in pyridine).
6.105 mg. substance: 5.335 mg. H_2O, 17.840 mg. CO_2.
6.788 " " : 5.940 " " 19.785 " "
$C_{30}H_{44}OS$. Calculated. C 79.60, H 9.82.
Found. (*a*) " 79.70, " 9.78.
(*b*) " 79.50, " 9.79.

The cholesterol test gave a cherry-red color which gave way to a yellowish green when the solution was warmed.

Dehydrogenation of β-Amyrin.—A mixture of 1.0 gm. of β-amyrin and an equal amount of sulfur was heated for 3 hours at a bath temperature of 200–210°. The reaction mass was extracted with ether and the ether solution was concentrated to a small volume and then filtered into a 10 cc. Claisen distilling flask. The residue was distilled at 2 mm. with the bath between 300–350°. The cherry-colored resin obtained in this manner was taken up in alcohol and the solution was decolorized with charcoal. The substance was precipitated from a boiling hot solution by addition of water. When this product was recrystallized from alcohol it separated in the form of fine needles which melted at 200–201°. The yield was 35 per cent.

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[9] Smith, J. H. C., and Young, W. G., *J. Biol. Chem.*, **76**, 289 (1927).

$[\alpha]_D^{25}$ = +86° (c = 1.020 in pyridine).
4.805 mg. substance: 4.222 mg. H_2O, 14.050 mg. CO_2.
3.665 " " : 3.243 " " 10.640 " "
7.432 " " : 3.885 " $BaSO_4$.
$C_{30}H_{44}OS$. Calculated. C 79.60, H 9.82, S 7.08.
Found. (a) " 79.74, " 9.83.
(b) " 79.17, " 9.90.
(c) S 7.18.

The cholesterol test gave a cherry-red color which on heating gave way to a yellowish green. No depression of melting point could be observed when this substance was mixed with that obtained by saponification of the above benzoate. The identity of the substances obtained was confirmed by comparison of their benzoates.

A solution of 0.1 gm. of $C_{30}H_{44}OS$, obtained from β-amyrin, in 1 cc. of benzene was refluxed with an excess of pyridine and benzoyl chloride for 1 hour. After evaporation on the steam bath, the residue was recrystallized from benzene by addition of methyl alcohol. The substance melted at 224–225° and showed no depression when mixed with the previous benzoate. In all other properties it proved to be identical.

$[\alpha]_D^{25}$ = +97° (c = 1.000 in pyridine).
4.040 mg. substance: 3.210 mg. H_2O, 11.820 mg. CO_2.
4.542 " " : 3.558 " " 13.330 " "
10.215 " " : 3.960 " $BaSO_4$.
$C_{37}H_{48}O_2S$. Calculated. C 79.80, H 8.70, S 5.75.
Found. (a) " 79.75, " 8.89.
(b) " 80.00, " 8.77.
(c) S 5.33.

Oxidation of the Benzoate, $C_{37}H_{48}O_2S$

A suspension of 5 gm. of the benzoate in 500 cc. of acetic acid was stirred vigorously while 190 cc. of 6 per cent $KMnO_4$ solution was added. After stirring for 2 hours complete solution occurred. This solution was then diluted with 1 liter of water and the precipitate was separated by filtration from the colloidal solution of MnO_2. 4.5 gm. of a mixture of benzoates were obtained, which was found to consist principally of the esters of the ketone $C_{30}H_{46}O_3$ and the lactone $C_{30}H_{44}O_4$. Although the separation of these substances was accomplished directly by fractionation from ligroin, it was found to be more advantageous to saponify the benzoates. For this purpose the crude substance was refluxed in 100 cc. of 10 per cent alcoholic KOH for 4 hours. After dilution, the resulting neutral precipitate was collected. The yield was 2.45 gm. When the alkaline filtrate was acidified, 1.1 gm. of an acid fraction was obtained. From the neutral fraction the ketone and lactone were separated as follows.

The Lactone, $C_{30}H_{44}O_4$.—Repeated recrystallizations from methyl alcohol of the above neutral fraction yielded homogeneous stout prisms which melted constantly at 299–300° with decomposition and were readily soluble in the usual solvents. Although this substance was recovered unchanged after prolonged heating at 130° with 5 per cent alcoholic KOH, more vigorous treatment as given below causes saponification to the acid, thus indicating its lactone character. It is not affected by concentrated hydrochloric acid at ordinary temperatures. The cholesterol test gave a reddish brown color which deepened when warmed.

$[\alpha]^{30}_D = -14°$ (c = 1.010 in pyridine).
4.540 mg. substance: 3.880 mg. H_2O, 12.855 mg. CO_2.
4.810 " " : 4.100 " " 13.570 " "
$C_{30}H_{44}O_4$. Calculated. C 76.90, H 9.48.
Found. (*a*) " 77.22, " 9.57.
(*b*) " 76.94, " 9.54.

The Acetate of the Lactone, $C_{30}H_{44}O_4$.—A solution of 0.05 gm. of the lactone and 0.05 gm. of fused sodium acetate in 2 cc. of acetic anhydride was refluxed for 7 hours. At the end of this period the solution was diluted with water and the collected precipitate was recrystallized from dilute alcohol. It crystallized in the form of shining plates which melted at 269–271°.

5.048 mg. substance: 4.105 mg. H_2O, 13.930 mg. CO_2.
4.600 " " : 3.670 " " 12.645 " "
$C_{32}H_{46}O_5$. Calculated. C 75.41, H 9.09.
Found. (*a*) " 75.26, " 9.10.
(*b*) " 74.97, " 8.93.

The Benzoate of the Lactone, $C_{30}H_{44}O_4$.—A solution of 0.05 gm. of the lactone in benzene was refluxed for 3 hours with an excess of pyridine and benzoyl chloride. The excess of reagents was removed by repeated evaporation with benzene. The residue after recrystallization from alcohol formed needles which melted at 292–293°.

3.917 mg. substance: 2.940 mg. H_2O, 11.178 mg. CO_2.
3.845 " " : 3.005 " " 10.930 " "
$C_{37}H_{48}O_5$. Calculated. C 77.60, H 8.47.
Found. (*a*) " 77.83, " 8.40.
(*b*) " 77.52, " 8.74.

The same benzoate was obtained directly by fractionation from ligroin of the crude mixture resulting from the oxidation of the benzoate of the thio compound. After repeated recrystallization from alcohol it formed needles which melted at 295–297° and showed no depression when mixed with the above benzoate.

3.992 mg. substance: 3.035 mg. H_2O, 11.312 mg. CO_2.
4.738 " " : 3.654 " " 13.485 " "
Found. (*a*) C 77.27, H 8.51.
(*b*) " 77.62, " 8.63.

The Acid, $C_{30}H_{46}O_5$.—A solution of 0.1 gm. of the lactone in 6 cc. of 10 per cent alcoholic KOH was heated in a sealed tube at 120–130° for 3 hours. The diluted mixture was acidified and the precipitate of acid and silica was collected and dried. The alcoholic solution was filtered from silica. On dilution of the filtrate, the crystalline acid separated as prisms. After recrystallization from acetone it melted at 237–238°.

4.055 mg. substance: 3.480 mg. H_2O, 11.030 mg. CO_2.
$C_{30}H_{46}O_5$. Calculated. C 74.01, H 9.54.
Found. " 74.19, " 9.60.

For the titration 12.702 mg. of the acid were dissolved in 2 cc. of alcohol and titrated with 0.1 N NaOH against phenolphthalein. Found, 0.276 cc. Calculated for 1 equivalent, 0.261 cc. After refluxing with an excess of alkali no further consumption was noted.

The same acid was obtained directly from the crude acid fraction formed on the saponification of the original oxidation mixture. This crude fraction on recrystallization from alcohol formed prisms which after being recrystallized from acetone melted at 238–239°.

$[\alpha]_D^{29}$ = +96° (c = 1.030 in pyridine).
3.912 mg. substance: 3.330 mg. H_2O, 10.580 mg. CO_2.
4.777 " " : 4.075 " " 12.922 " "
$C_{30}H_{46}O_5$. Calculated. C 74.01, H 9.54.
Found. (*a*) " 73.76, " 9.53.
(*b*) " 73.78, " 9.55.

8.575 mg. of substance were dissolved in 2 cc. of alcohol and directly titrated with 0.1 N NaOH against phenolphthalein. Found, 0.200 cc. Calculated for 1 equivalent, 0.177 cc.

The Ketone, $C_{30}H_{46}O_3$.—This substance was isolated from the crude methyl alcoholic mother liquors obtained from the above lactone. After repeated recrystallization from ether it formed long slender needles which melted at 274–275°.

$[\alpha]_D^{26}$ = −127° (c = 1.023 in pyridine).
4.285 mg. substance: 3.998 mg. H_2O, 12.455 mg. CO_2.
4.700 " " : 4.264 " " 13.670 " "
$C_{30}H_{46}O_3$. Calculated. C 79.30, H 10.22.
Found. (*a*) " 79.27, " 10.43.
(*b*) " 79.32, " 10.15.

This substance may be recovered from its solution in concentrated hydrochloric acid unchanged. The cholesterol test gave a yellow-orange color which changed to amber when the solution was warmed.

The Oxime of the Ketone, $C_{30}H_{46}O_3$.—A solution of 0.1 gm. of the ketone in alcohol was refluxed for 5 hours with twice the calculated amounts of sodium acetate and hydroxylamine hydrochloride. The solution on dilution with water gave a precipitate which was recrystallized from acetone. It separated in the form of prisms which melted at 234–236° with decomposition.

3.820 mg. substance: 3.385 mg. H_2O, 10.740 mg. CO_2.
7.557 " " : 0.220 cc. N (29°, 756.0 mm.).
$C_{30}H_{47}O_3N$. Calculated. C 76.69, H 10.08, N 3.00.
Found. (*a*) " 76.70, " 9.92.
(*b*) N 3.27.

The Acetate of the Ketone, $C_{30}H_{46}O_3$.—A solution of equal amounts of the ketone and fused sodium acetate in acetic anhydride was refluxed for 7 hours. The solution was diluted with water and the resulting substance was recrystallized from alcohol by dilution with water. The melting point observed was 231–232°. The low hydrogen figures obtained on analysis of this substance were not further investigated.

4.605 mg. substance: 3.908 mg. H_2O, 13.045 mg. CO_2.
4.390 " " : 3.670 " " 12.403 " "
$C_{32}H_{48}O_4$. Calculated. C 77.36, H 9.75.
Found. (*a*) " 77.25, " 9.50.
(*b*) " 77.06, " 9.35.

The Benzoate of the Ketone, $C_{30}H_{46}O_3$.—A benzene solution of 0.2 gm. of the ketone was refluxed for 1 hour with an excess of pyridine and benzoyl chloride. After evaporation, the residue was taken up in alcohol and precipitated with water. The benzoate separated as needles when recrystallized from alcohol and melted at 261–262°.

$[\alpha]_D^{24} = -77°$ (c = 1.003 in pyridine).
2.920 mg. substance: 2.365 mg. H_2O, 8.518 mg. CO_2.
4.625 " " : 3.657 " " 13.478 " "
$C_{37}H_{50}O_4$. Calculated. C 79.51, H 9.02.
Found. (*a*) " 79.56, " 9.06.
(*b*) " 79.48, " 8.85.

This benzoate proved to be resistant to the oxidizing action of permanganate in acetic acid solution. Apparently the same benzoate, although not quite pure, was obtained directly from the mother liquor after removal of the benzoate of the lactone from the mixture of crude benzoates which resulted from the oxidation of the sulfur compound. Repeated recrystallization from alcohol finally yielded needles which melted at 255–257°, somewhat lower than the above benzoate. When mixed no further depression of the melting point was observed.

4.109 mg. substance: 3.153 mg. H_2O, 11.897 mg. CO_2.
4.035 " " : 3.200 " " 11.660 " "
Found. (a) C 78.98, H 8.59.
(b) " 78.81, " 8.88.

Clemmensen Reduction of the Ketone, $C_{30}H_{46}O_3$.—A solution of 0.05 gm. of the ketone in 5 cc. of acetic acid was treated with 2 gm. of amalgamated zinc and 1 cc. of hydrochloric acid (1.19). The mixture was refluxed for 1½ hours and then precipitated with water. When recrystallized from dilute alcohol, the desoxy derivative melted not sharply at 155–160°. It is to be doubted that the substance was homogeneous and owing to the limited amount of material available it was not investigated further.

4.252 mg. substance: 4.135 mg. H_2O, 12.610 mg. CO_2.
4.480 " " : 4.460 " " 13.267 " "
$C_{30}H_{48}O_2$. Calculated. C 81.75, H 10.98.
Found. (a) " 80.89, " 10.88.
(b) " 80.77, " 11.14.

Hydrogenation of the Ketone, $C_{30}H_{46}O_3$.—0.2 gm. of the ketone in alcoholic solution was hydrogenated with 0.1 gm. of platinum oxide catalyst. After reduction of the catalyst the hydrogen absorption proceeded slowly but steadily. Since after 18 hours the addition of more catalyst caused no further absorption, the operation was discontinued. The volume of 40 cc. noted was approximately the equivalent of 4 mols of hydrogen. On concentration after removal of the catalyst, the substance crystallized as needles which melted at 205–215°. This material was probably a mixture of isomers and after repeated recrystallization from alcohol it melted at 200–202°. The analytical figures approximated those for amyrin.

3.700 mg. substance: 3.745 mg. H_2O, 11.405 mg. CO_2.
3.845 " " : 3.835 " " 11.815 " "
3.933 " " : 4.000 " " 12.080 " "
$C_{30}H_{50}O$. Calculated. C 84.45, H 11.73.
Found. (a) " 84.07, " 11.33.
(b) " 83.81, " 11.17.
(c) " 83.77, " 11.38.

In a second hydrogenation experiment a similar substance was obtained which on repeated recrystallization from methyl alcohol melted not sharply from 207–226° and was apparently a mixture of stereoisomers.

4.437 mg. substance: 4.425 mg. H_2O, 13.620 mg. CO_2.
Found. C 83.72, H 11.16.

This substance did not react with hydroxylamine but yielded a benzoate. This proved to be a mixture of isomers which melted from 205–215°. The analytical figures, however, with several samples of repeatedly recrystallized benzoate gave analytical figures which were in consistent disagreement with the calculated figures for a monobenzoate of $C_{30}H_{50}O$. The following is representative of these results.

4.258 mg. substance: 3.628 mg. H_2O, 12.950 mg. CO_2.
$C_{37}H_{54}O_2$. Calculated. C 83.71, H 10.26.
Found. " 82.94, " 9.54.

Partial Dehydrogenation of α-Amyrin and Benzoate

Dehydrogenation of α-Amyrin Benzoate.—A mixture of 6.0 gm. of α-amyrin benzoate and an equal amount of sulfur was heated in an atmosphere of nitrogen for 6 hours at a bath temperature of 220–230°. Considerable carbonization occurred. The reaction mass was extracted with ether and the filtered solution was evaporated to dryness. When ether was again added to the residue complete solution did not take place. This undissolved sulfur was removed by filtration and the solution was again evaporated to dryness. This process was repeated until addition of ether produced complete solution. The final ether-soluble product from this treatment was dissolved in alcohol and the solution was decolorized with carbon. On concentration, dehydro-α-amyrin benzoate crystallized in the form of shining plates. The product was recrystallized from alcohol and then from acetone. 0.6 gm. of substance was obtained which melted at 171–172°. The cholesterol test proved to be quite characteristic and of a different character from that shown by α- and β-amyrin benzoates. This benzoate gave a light blue color which deepened to an indigo blue when the solution was warmed for a short time.

$[\alpha]_D^{23} = +304°$ (c = 1.023 in pyridine).
5.237 mg. substance: 4.600 mg. H_2O, 16.115 mg. CO_2.
4.175 " " : 3.718 " " 12.813 " "
$C_{37}H_{52}O_2$. Calculated. C 84.03, H 9.92.
Found. (*a*) " 83.92, " 9.83.
(*b*) " 84.10, " 10.00.

Dehydro-α-Amyrin Benzoate from α-Amyrin.—A mixture of 1.7 gm. of α-amyrin and an equal amount of sulfur was heated in an atmosphere of nitrogen for 5 hours at a bath temperature of 200–210°. The mass was extracted with ether and the sulfur removed as in the case of the above benzoate. It was found advantageous to convert this product into the benzoate for purposes of purification. Accordingly, it was dissolved in benzene and was refluxed for 1 hour with benzoyl chloride and pyridine. The excess reagents were removed by repeated evaporation with benzene. The resulting syrup was taken up in alcohol and after treatment with norit the solution was concentrated on the steam bath until crystallization took place. The resulting substance was identical with the dehydro-α-amyrin benzoate described above. It melted at 171–172°.

$[\alpha]_D^{23} = +305°$ (c = 1.007 in pyridine).
4.902 mg. substance: 4.370 mg. H_2O, 15.110 mg. CO_2.
$C_{37}H_{52}O_2$. Calculated. C 84.03, H 9.92.
Found. " 84.06, " 9.98.

Dehydro-α-Amyrin.—The benzoate was refluxed with 10 parts of 10 per cent alcoholic KOH for 5 hours. The hot solution was diluted with water. The resulting precipitate was collected with water and recrystallized from hot acetone by dilution with water. It crystallized in the form of long, slender needles which melted at 162–163°. The cholesterol test gave an immediate deep indigo-blue in the cold.

$[\alpha]_D^{23} = +358°$ (c = 1.000 in pyridine).
4.202 mg. substance: 4.278 mg. H_2O, 13.085 mg. CO_2.
3.977 " " : 3.950 " " 12.385 " "
$C_{30}H_{48}O$. Calculated. C 84.91, H 11.32.
Found. (*a*) " 84.92, " 11.39.
(*b*) " 84.93, " 11.12.

Dehydro-α-Amyrone.—A solution of 0.2 gm. of dehydro-α-amyrin in 5 cc. of acetic acid was oxidized by slow addition of an acetic acid solution of 0.07 gm. of CrO_3. This solution was refluxed for ½ hour and then diluted while hot. The resulting substance when recrystallized from methyl alcohol separated in the form of plates which melted at 133–134°. The cholesterol test gave a light violet color which deepened when the solution was warmed and then gradually changed to a green.

$[\alpha]_D^{20} = +412°$ (c = 0.870 in pyridine).
3.964 mg. substance: 3.860 mg. H_2O, 12.310 mg. CO_2.
4.450 " " : 4.402 " " 13.833 " "
4.125 " " : 4.138 " " 12.915 " "
$C_{30}H_{46}O$. Calculated. C 85.20, H 10.73.
Found. (a) " 84.70, " 10.90.
(b) " 84.78, " 11.07.
(c) " 85.39, " 11.22.

α-Amyrone.—For comparison α-amyrone was prepared according to Zinke.[10] It possessed the recorded properties and melted at 124° after preliminary sintering. The cholesterol test gave a light violet color which gradually faded to a yellow when warmed.

$[\alpha]_D^{25} = +119°$ (c = 1.007 in pyridine).
4.558 mg. substance: 4.572 mg. H_2O, 14.185 mg. CO_2.
4.733 " " : 4.833 " " 14.690 " "
$C_{30}H_{48}O$. Calculated. C 84.91, H 11.32.
Found. (a) " 84.87, " 11.23.
(b) " 84.65, " 11.42.

Dehydro-α-Amyroneoxime.—A methyl alcoholic solution of 0.05 gm. of dehydro-α-amyrone was refluxed with an excess of hydroxylamine hydrochloride and sodium acetate for 2 hours. The oxime was precipitated from the boiling solution by the addition of water. When recrystallized from methyl alcohol it separated in the form of needles which melted at 233–235°.

4.332 mg. substance: 4.200 mg. H_2O, 13.120 mg. CO_2.
2.230 " " : 2.110 " " 6.743 " "
4.728 " " : 0.136 cc. N (26°, 775.9 mm.).
$C_{30}H_{47}ON$. Calculated. C 82.30, H 10.84, N 3.20.
Found. (a) " 82.60, " 10.85.
(b) " 82.47, " 10.60.
(c) N 3.35.

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[10] Zinke, A., *Monatsh. Chem.*, **42**, 439 (1924).

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SAPONINS

V. THE PARTIAL DEHYDROGENATION OF HEDERAGENIN

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Simultaneously with the experiments on the partial dehydrogenation with sulfur of the amyrins,[1] we have also carried on analogous studies with hederagenin derivatives. In preliminary experiments with hederagenin methyl ester a relatively small amount of a crystalline sulfur compound was obtained, but since much better results were secured with another derivative the investigation of this substance was discontinued. With the idea that the free hydroxyl groups of hederagenin methyl ester contributed perhaps to the very poor yield of sulfur derivative, we have turned to another substance in which these groups have been removed. The derivative selected was the previously described substance $C_{31}H_{50}O_2$,[2] which was prepared by the Clemmensen reduction of the ketone, $C_{31}H_{48}O_3$. This ketone had been in turn obtained by oxidative degradation of hederagenin methyl ester. For convenience of designation we propose the name hedragonic methyl ester for this ketone and for its reduction product, $C_{31}H_{50}O_2$, the name hedraganic methyl ester. When the latter substance was partially dehydrogenated with sulfur, about 50 per cent of a crystalline sulfur-containing derivative was obtained in which the methyl ester group remained intact. The formula of this substance suggested by the analytical figures is $C_{31}H_{44}O_2S$ which must have been formed by the removal of 3 mols of hydrogen and the introduction of 1 sulfur atom. This derivative proved to be analogous to the sulfur compound obtained from β-amyrin.

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[1] Jacobs, W. A., and Fleck, E. E., *J. Biol. Chem.*, **88**, 137 (1930).

[2] Jacobs, W. A., and Gustus, E. L., *J. Biol. Chem.*, **69**, 641 (1926).

The sulfur atom resisted treatment with alkali and is unquestionably of cyclic character. The analogy of this substance to the β-amyrin derivative was further shown by its behavior on oxidation with permanganate in acetic acid solution. Here again two neutral sulfur-free oxidation products were formed, which were shown to be respectively $C_{31}H_{46}O_4$ and $C_{31}H_{44}O_5$ and were apparently analogous to the substances obtained from the β-amyrin thio compound. The former proved to be a ketone since it yielded a monooxime. Attempts to acylate this ketone were not successful, leaving for the moment the nature of the other oxygen atom undetermined. The separation of this substance from the other oxidation products afforded considerable difficulty which probably accounts for the deviation from the theory of the analytical data obtained from it and its derivatives. The second oxidation product, also of neutral character, did not yield an oxime and could not be acylated but was shown by its behavior towards alkali to be in all likelihood a lactone. But in the study of the action of alkali on these substances it was found necessary to take into consideration a complicating factor in the form of the methyl ester group.

In previous papers on hederagenin methyl ester and its derivatives, the stability and resistance to saponification of this ester group have been reported.[3] In view of the observations given below, we have studied again the behavior towards alkali of hederagenin methyl ester itself and certain of its derivatives. Although as originally reported, the ester group of these substances is unusually resistant to the saponifying action of alkali, conditions have now been found for their saponification. This was accomplished by heating the esters for 3 to 5 hours in 10 per cent alcoholic potassium hydroxide at 130°. It was possible then to isolate in each case about 50 per cent of the free acids along with unchanged ester.

On turning to the study of the action of alkali on the thio compound described above, the resistant property of the methyl ester group was found to be retained. In contrast to this, however, the methyl ester groups of the two oxidation products derived from the sulfur compound were found to be surprizingly easily saponified.

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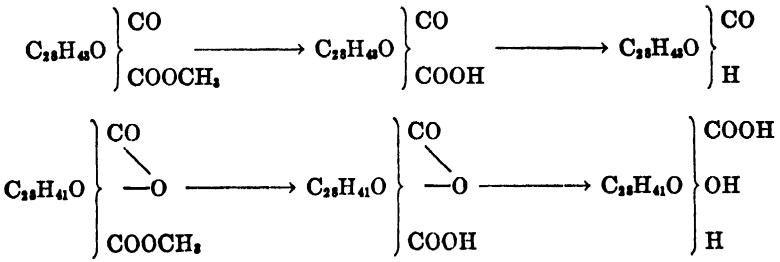
[3] Jacobs, W. A., *J. Biol. Chem.*, **63**, 628 (1925). van der Haar, A. W., *Rec. trav. chim. Pays-Bas*, **44**, 740 (1925).

This was quantitatively accomplished by refluxing these substances in dilute alcoholic alkali which was the equivalent of 0.05 N. From each substance, $C_{31}H_{46}O_4$ and $C_{31}H_{44}O_5$, were obtained the free acids, $C_{30}H_{44}O_4$ and $C_{30}H_{42}O_5$, respectively. Since the sulfur compound itself cannot be similarly saponified, it is apparent that the sulfur atom must be situated in the same general vicinity in the molecule as the carbomethoxyl group in order that its removal on oxidation with the introduction of oxygen atoms should labilize this ester group. This conclusion was further supported by the observation that the carboxyl group itself which was originally present in hederagenin and is now liberated again by saponification of the ester group displays a new kind of lability not previously noted in hederagenin and its derivatives. On heating the above keto ester, $C_{31}H_{46}O_4$, with 10 per cent potassium hydroxide at 130°, a neutral substance was obtained which was found to possess the formula $C_{29}H_{44}O_2$. This substance owed its origin to the loss of CO_2 from the carboxyl group following its preliminary liberation by saponification from the ester group.

The second oxidation product, $C_{31}H_{44}O_5$, which has been described as a lactone, exhibitea a similar behavior but in this case the lactone group was simultaneously saponified. Therefore, the opening of the lactone group together with saponification of the methyl ester group and simultaneous loss of CO_2 from the carboxyl group liberated in the latter case resulted in an acid substance, $C_{29}H_{44}O_4$. In the case of the keto ester and the lactone ester these reactions may be represented as follows:



$$C_{28}H_{43}O\left\{\begin{array}{l}CO\\ \\ COOCH_3\end{array}\right. \longrightarrow C_{28}H_{43}O\left\{\begin{array}{l}CO\\ \\ COOH\end{array}\right. \longrightarrow C_{28}H_{43}O\left\{\begin{array}{l}CO\\ \\ H\end{array}\right.$$

$$C_{28}H_{41}O\left\{\begin{array}{l}CO\\ \quad\diagdown\\ \;—O\\ \\ COOCH_3\end{array}\right. \longrightarrow C_{28}H_{41}O\left\{\begin{array}{l}CO\\ \quad\diagdown\\ \;—O\\ \\ COOH\end{array}\right. \longrightarrow C_{28}H_{41}O\left\{\begin{array}{l}COOH\\ \\ OH\\ \\ H\end{array}\right.$$

The formation of these substances, therefore, involves the loss of the original carboxyl group of hederagenin.

The lability of the ester carboxyl in these oxidation products suggests that the ketone $C_{31}H_{46}O_4$, is a β-keto ester and that in the ester lactone, $C_{31}H_{44}O_5$, the lactone group is also in a β position to the methyl ester group. From these facts it may be suggested with reservation that in the thio compound one of the carbon atoms to which the sulfur atom is attached is β to the methyl ester group. On removal of the sulfur atom by oxidation, this carbon atom now becomes carbonyl in the case of keto ester and a lactone carbon in the case of the second oxidation product. Although it is possible to suggest explanations of the formation of such oxidation products from an assumed polycyclic thiophene derivative, we do not feel that this is justified until more data have been obtained.

The formation of the sulfur compounds and their behavior on oxidation in both β-amyrin and hedraganic methyl ester present exactly analogous pictures which very strongly suggest that identical structures are involved in each case. A study of this reaction is being extended to include other sapogenins, triterpene alcohols, etc.

EXPERIMENTAL

Dehydrogenation of Hedraganic Methyl Ester.—A mixture of 5 gm. of hedraganic methyl ester[2] and an equal amount of sulfur was heated in an atmosphere of nitrogen for 5 hours, at a temperature of 210–220°. The reaction mass was extracted with ether and the filtered solution was evaporated to dryness. The resulting solid was distilled at 3 mm. from a 10 cc. Claisen distilling flask. The temperature of the metal bath, in which the flask was immersed, was gradually increased until a temperature of 275° was attained. At this point practically all of the free sulfur had distilled. A cherry-colored resin distilled when the temperature of the bath was maintained between 300–350°. A solution of this resin in 10 cc. of hot acetone gradually crystallized. The collected substance was then recrystallized from acetone and finally from alcohol. It separated in the form of colorless prisms which melted at 137–138°. In pyridine solution no appreciable optical activity could be noted.

For analysis the substance was recrystallized four times from acetone. Following this, in order to confirm the analytical data the substance was again recrystallized four times from acetone.

(*a*) 5.880 mg. substance*: 4.700 mg. H_2O, 16.665 mg. CO_2.
(*b*) 4.668 " " *: 3.837 " " 13.260 " "
(*c*) 4.508 " " †: 3.740 " " 12.810 " "
(*d*) 5.220 " " †: 4.210 " " 14.810 " "
(*e*) 7.425 " " : 3.460 mg. $BaSO_4$.

$C_{31}H_{44}O_2S$. Calculated. C 77.45, H 9.16, S 6.67.
$C_{30}H_{42}O_2S$. " " 77.20, " 9.08, " 6.86.
Found. (*a*) " 77.30, " 8.95.
(*b*) " 77.46, " 9.19.
(*c*) " 77.50, " 9.28.
(*d*) " 77.38, " 9.03.
(*e*) S 6.43.

* Recrystallized four times.
† Recrystallized eight times.

Titration showed no free acidity. In an experiment in which the substance was refluxed for 5 hours with 1 per cent alcoholic KOH, 50 per cent of recrystallized starting material was recovered and only an inappreciable amount of acid product was obtained. The methyl ester group, as in the starting material, is thus seen to be difficult to saponify. The cholesterol test gave an orange solution which changed to a deep red when warmed. A molecular weight determination according to the method of Rast gave the following result:

14.587 mg. camphor: 2.242 mg. substance, $\Delta = 14.5°$. Mol. wt., calculated, 476.4; found, 450.

Oxidation of the Thio Compound

The Lactone Ester, $C_{31}H_{44}O_5$.—A solution of 3 gm. of the above sulfur compound in 300 cc. of acetic acid was stirred while 125 cc. of 6 per cent $KMnO_4$ solution were added. After stirring for 1 hour, the solution was diluted with 3 volumes of water. The white precipitate which separated was removed from the colloidal solution of MnO_2 by filtration. Final traces of MnO_2 were eliminated by dissolving the precipitate in alcohol and filtering the solution. The alcoholic filtrate was evaporated to dryness and the residue was dissolved in ligroin (80–90°). This solution was then concentrated on a steam bath to a volume of 300 cc. The cooled solution deposited long, needle-shaped crystals. When this product was recrystallized from acetone it melted at 274–275°. The cholesterol test gave a yellow color which deepened to an amber when the solution was warmed.

$[\alpha]_D^{25} = -16°$ (c = 1.017 in pyridine).
5.130 mg. substance: 4.022 mg. H_2O, 14.070 mg. CO_2.
4.262 " " : 3.400 " " 11.642 " "
5.490 " " : 2.692 " AgI.
$C_{31}H_{44}O_5$. Calculated. C 74.96, H 8.92, OCH_3 6.25.
$C_{30}H_{42}O_5$. " " 74.70, " 8.72, " 6.44.
Found. (*a*) " 74.80, " 8.77.
(*b*) " 74.52, " 8.92.
(*c*) OCH_3 6.

Contrary to the original thio compound and hederagenin methyl ester, the ester group of this oxidation product is easily saponified by dilute alkali as follows:

Saponification of the Methyl Ester Group of the Lactone Ester, $C_{31}H_{44}O_5$.—15.372 mg. of substance were dissolved in 4 cc. of alcohol. The solution showed no free acidity. 3.203 cc. of 0.1 N NaOH were added and the mixture was refluxed in an atmosphere of nitrogen for 4 hours, and then titrated back. Found, 0.239 cc. Calculated for 1 equivalent, 0.262 cc.

The solution on acidification with acetic acid and concentration yielded the crystalline lactone acid. When this product was recrystallized from diluted acetone, it separated in the form of plates which melted at 255–256° with decomposition.

4.370 mg. substance: 3.380 mg. H_2O, 11.900 mg. CO_2.
$C_{30}H_{42}O_5$. Calculated. C 74.62, H 8.79.
$C_{29}H_{40}O_5$. " " 74.31, " 8.60.
Found. " 74.27, " 8.65.

Cleavage of the Lactone Ester, $C_{31}H_{44}O_5$, with Strong Alkali.—A solution of 0.2 gm. of the lactone ester in 15 cc. of 10 per cent alcoholic KOH was heated in a sealed tube at 130–140° for 4 hours. The solution was diluted with 3 volumes of water and the small amount of precipitate which separated was filtered off. The filtrate was acidified and the acid product which precipitated was collected with water. When recrystallized from acetone it separated in the form of hexagonal plates which melted at 209–210°.

4.900 mg. substance: 4.265 mg. H_2O, 13.585 mg. CO_2.
3.660 " " : 3.070 " " 10.180 " "
$C_{29}H_{44}O_4$. Calculated. C 76.25, H 9.73.
$C_{28}H_{42}O_4$. " " 75.95, " 9.58.
Found. (*a*) " 75.61, " 9.74.
(*b*) " 75.86, " 9.39.

10.355 mg. substance were dissolved in 4 cc. of alcohol and directly titrated with 0.1 N NaOH against phenolphthalein. Found, 0.250 cc. Calculated for 1 equivalent, 0.227 cc.

In attempts to prepare a benzoate and an oxime of the lactone ester it was recovered unchanged.

The Keto Ester, $C_{31}H_{46}O_4$.—This product of the oxidation of the thio compound was obtained from the ligroin mother liquor of the first crystallization of the above lactone ester. When this solution was concentrated to about 25 cc. long, fibrous needles separated on cooling. Purification of this substance was accomplished by repeated fractionation from ether, in which the lactone ester is much less soluble. This separation proved to be very difficult and it is possible that the substance was still not homogeneous. This may account in part for the rather unsatisfactory analytical figures obtained with this and its succeeding derivatives. Final purification was attempted by dissolving the substance in ether and inducing crystallization by the addition of petrolic ether. The silky needles obtained melted at 202–204°.

$[\alpha]_D^{28} = -184°$ (c = 1.027 in pyridine).
5.420 mg. substance: 4.385 mg. H_2O, 15.240 mg. CO_2.
4.280 " " : 3.470 " " 12.055 " "
5.160 " " : 2.505 " AgI.

$C_{31}H_{46}O_4$.	Calculated.		C 77.11,	H 9.62,	OCH_3 6.44.
$C_{30}H_{44}O_4$.	"		" 76.85,	" 9.41,	" 6.63.
	Found.	(*a*)	" 76.69,	" 9.05.	
		(*b*)	" 76.83,	" 9.06.	
		(*c*)			OCH_3 6.41.

In an attempt to benzoylate the keto ester it was recovered unchanged.

The Oxime of the Keto Ester, $C_{31}H_{46}O_4$.—A mixture of 0.1 gm. of the keto ester and hydroxylamine in absolute alcoholic solution was heated in a sealed tube at a temperature of 110–120° for 10 hours. The alcoholic solution was then concentrated and the oxime was allowed to crystallize. Repeated recrystallizations from alcohol gave a substance which melted at 236–237° with decomposition.

4.730 mg. substance: 3.940 mg. H_2O, 13.045 mg. CO_2.
4.000 " " : 3.200 " " 11.045 " "
7.450 " " : 0.196 cc. N (26°, 761.5 mm.).
4.903 " " : 2.560 mg. AgI.
$C_{31}H_{47}O_4N$. Calculated. C 74.79, H 9.54, N 2.82, OCH_3 6.23.
$C_{30}H_{45}O_4N$. " " 74.47, " 9.39, " 2.89, " 6.41.
Found. (*a*) " 75.22, " 9.32.
(*b*) " 75.28, " 8.95.
(*c*) N 3.00.
(*d*) OCH_3 6.89.

Saponification of the Methyl Ester Group of the Keto Ester, $C_{31}H_{46}O_4$.—10.867 mg. of substance were dissolved in 4 cc. of alcohol. The solution showed no free acidity. 3.222 cc. of 0.1 N NaOH were added and the solution was refluxed in an atmosphere of nitrogen for 4 hours and then titrated back. Found, 0.252 cc. Calculated for 1 equivalent, 0.242 cc.

When the solution was acidified with acetic acid and then concentrated, crystallization occurred. When this product was recrystallized from dilute acetone it separated in the form of fine needles which melted at 184–185°with decomposition.

3.987 mg. substance: 3.270 mg. H_2O, 11.222 mg. CO_2.
3.955 " " : 3.110 " " 11.167 " "
$C_{30}H_{44}O_4$. Calculated. C 76.85, H 9.41.
$C_{29}H_{42}O_4$. " " 76.60, " 9.32.
Found. (*a*) " 76.76, " 9.18.
(*b*) " 77.00, " 8.80.

Cleavage of the Keto Ester, $C_{31}H_{44}O_4$, with Strong Alkali.—A solution of 0.2 gm. of the ketone in 15 cc. of 10 per cent alcoholic potassium hydroxide solution was heated in a sealed tube at 130–140° for 3½ hours. The solution was diluted with 3 volumes of water and the precipitate was collected with water. The dried material was extracted with a small amount of petroleum ether in order to remove a small amount of non-crystalline by-product. When the residue was recrystallized from ether it separated in the form of needles which melted at 262–264° and proved to be neutral.

4.100 mg. substance: 3.660 mg. H_2O, 12.415 mg. CO_2.
3.595 " " : 3.170 " " 10.900 " "
$C_{29}H_{42}O_2$. Calculated. C 82.41, H 10.03.
$C_{28}H_{40}O_2$. " " 82.35, " 9.88.
Found. (*a*) " 82.61, " 9.99.
(*b*) " 82.69, " 9.87.

Saponification of Hederagenin Methyl Ester.—A solution of 0.2 gm. of the ester in 10 cc. of 10 per cent alcoholic KOH was heated in a sealed tube at 120–130° for 5 hours. Dilution with 3 volumes of water gave a precipitate which was collected and dried. The dried material was extracted with ether, leaving the insoluble potassium salt of hederagenin. The latter was obtained by liberation with acid and recrystallization from alcohol. The resulting substance agreed in all properties with hederagenin.

4.050 mg. substance: 3.800 mg. H_2O, 11.358 mg. CO_2.
$C_{31}H_{50}O_4$. Calculated. C 76.48, H 10.36.
Found. " 76.49, " 10.50.

The ether extract of the above potassium salt yielded 60 mg. of residue which after recrystallization was shown to be unchanged starting material.

Saponification of Hedraganic Methyl Ester.—A solution of 0.1 gm. of the methyl ester in 10 cc. of 10 per cent alcoholic KOH was heated at 120–130° for 3 hours. The solution was then diluted with 3 volumes of water and the precipitate was collected with water. The dried precipitate was extracted with dry ether. The insoluble residue of potassium salt was suspended in ether and shaken with dilute HCl. When complete solution had occurred due to the liberation of the free acid, the ether solution was washed repeatedly with water and then concentrated. The residue was recrystallized from acetone by the addition of water. Hedraganic acid formed needles which melted at 242–243°.

2.540 mg. substance: 2.440 mg. H_2O, 7.637 mg. CO_2.
$C_{30}H_{48}O_2$. Calculated. C 81.75, H 10.99.
Found. " 82.00, " 10.72.

The above ether extract of the potassium salt on evaporation gave a neutral residue which was recrystallized from acetone. This fraction was identified by melting point and mixed melting point as starting material.

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TIGOGENIN, A DIGITALIS SAPOGENIN

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Digitonin and in smaller amount gitonin are the principal saponins of digitalis seeds. In recent work Windaus (1) and coworkers have made observations which indicate that still other saponins must be present. Although the latter were not isolated as such their presence was detected by the isolation of characteristic degradation products. When the pure sapogenins, digitogenin or gitogenin, were oxidized with chromic acid only acid substances, digitogenic acid and gitogenic acid were obtained respectively, with no neutral by-products. If, however, a digitogenin was used which had been obtained from crude digitonin, then an appreciable amount of neutral fraction was secured. Most of this consisted of a diketone, $C_{26}H_{38}O_4$, which was presumably an oxidation product of a dihydroxy compound, $C_{26}H_{42}O_4$, an isomer of gitogenin and in which the hydroxyl groups are not vicinal as in gitogenin. In still much smaller amount a neutral monoketone was obtained which was apparently an oxidation product of the secondary alcoholic group of still another sapogenin, $C_{26}H_{42}O_3$, and therefore isomeric with sarsapogenin. The sapogenins themselves were not isolated.

These conclusions have been substantiated in the case of the sapogenin, $C_{26}H_{42}O_3$, by the following observations which we have been able to make. In connection with one of our problems we have had occasion to prepare gitogenin by the hydrolysis of the saponin which we had collected in the course of the preparation of the cardiac glucosides from the leaves of *Digitalis purpurea*. Crude gitonin was found to crystallize in characteristic leaflets on concentration of the purified 50 per cent alcoholic extract of the leaves. When the crude saponin was hydrolyzed lting sapogenin was found to melt too low for gitoge ie analytical figures were consistently high in carbon. n found possible to separate the contaminant from g oy taking advantage of the greater solubility of the former in petrolic ether. By this means gitogenin was readily obtained in pure form. The more soluble substance was also readily purified and on analysis proved to possess the formula $C_{26}H_{42}O_3$. Thi. substance, which may be conveniently called *tigogenin,* proved t be a secondary alcohol yielding on oxidation a ketone which is probably identical with the ketone, $C_{26}H_{40}O_3$, of Windaus and Willerding. *Tigogenone* yielded a monooxime. Tigogenin gave a *monoacetate* and a *monobenzoate.* As in the case of digitogenin and gitogenin, the remaining two oxygens cannot be characterized directly and appear to be of oxidic character.

A comparison of sarsapogenin from the saponin of sarsaparilla root as well as its ketone sarsapogenone with tigogenin and its derivatives has shown that these sapogenins are isomeric. The substances which resulted by the reduction of the ketones according to the method of Clemmensen also proved to be different.

EXPERIMENTAL

Tigogenin.—2 kilos of *Digitalis purpurea* leaves, after preliminary extraction with water, were extracted twice with 6 liters of 50 per cent alcohol. The alcoholic extracts were precipitated with basic lead acetate, and the filtrate was freed from the excess of lead by careful addition of ammonium sulfate. The filtrate from $PbSO_4$ was concentrated under diminished pressure to remove the alcohol. The shining platelets which separated were collected with water. This product, which contained considerable colored impurity, was dissolved in a mixture of 500 cc. of 50 per cent alcohol and 50 cc. of HCl (1.19). The solution was refluxed for 3 hours and on cooling a dark green product was obtained. This was collected with water and then dissolved in 100 cc. of alcohol and 15 cc. of concentrated HCl and the solution was again heated for 3 hours. The crystals which separated on dilution with water were repeatedly recrystallized from a small volume of alcohol until the product was freed from colored impurities. This procedure yielded 1.3 gm of a mixture of tigogenin and gitogenin which melted between 240 and 250°. An additional amount of this mixture of genins was obtained by extraction of the lead sulfate precipitate with 50 per cent alcohol and working up this extract in a manner similar to that outlined above.

A chloroform solution saturated with this mixture at room temperature was precipitated by the addition of 2 volumes of petrol ether (b.p. 40–60°). This precipitate consisted of gitogenin which was still slightly contaminated since it melted at 260–262°.

4.800 mg. substance: 4.280 mg. H_2O, 13.100 mg. CO_2.
$C_{26}H_{42}O_4$. Calculated. C 74.58, H 10.12.
Found. " 74.43, " 9.98.

The mother liquor was evaporated to dryness and the process was repeated until no more gitogenin could be obtained. The final residue was recrystallized several times from acetone from which it separated in the form of prisms which melted at 203–204°. About 30 to 40 per cent of the mixture of genins was found to be tigogenin.

Tigogenin is soluble in all of the ordinary solvents. It proved to be more soluble in acetone, ether, ligroin (b. p. 80–90°), and petrol ether (b.p. 40–60°) than is gitogenin. This difference in solubility is most marked in the case of petrol ether (b.p. 40–60°).

The cholesterol test gave a light yellow color which turned to a reddish brown when the solution was warmed.

$[\alpha]_D^{25} = -49$ (c = 1.023 in pyridine).

For analysis the substance was dried at 100° and 30 mm.

4.856 mg. substance: 4.632 mg. H_2O, 13.847 mg. CO_2.
4.907 " " : 4.705 " " 13.950 " "
$C_{26}H_{42}O_3$. Calculated. C 77.55, H 10.52.
Found. (*a*) " 77.77, " 10.67.
(*b*) " 77.53, " 10.72.

The molecular weight determination was made according to the method of Rast.

30.547 mg. camphor: 3.118 mg. anhydrous substance, $\Delta = 11.75$. Mol. wt. calculated, 402.3; found, 382.

Tigogenin Acetate.—A solution of 0.2 gm. of tigogenin and an equal amount of fused sodium acetate in acetic anhydride was

refluxed for 5 hours. After dilution with water te
separated in the form of shining plates. After recr 'n
from alcohol it melted at 200–202°.

$[\alpha]_D^{24} = -57$ (c = 1.023 in pyridine).

4.365 mg. substance: 3.912 mg. H_2O, 12.110 mg. CO_2.
4.880 " " : 4.450 " " 13.570 " "
$C_{28}H_{44}O_4$. Calculated. C 75.62, H 9.97.
Found. (a) " 75.67, " 10.03.
(b) " 75.83, " 10.20.

Tigogenin Benzoate.—A benzene solution of 0.1 gm. of tigogenin was refluxed for 2 hours with an excess of benzoyl chloride and pyridine. After concentrating the washed benzene solution, the residue was recrystallized from acetone. The benzoate separated in the form of plates which melted at 224–225°.

$[\alpha]_D^{26} = -37$ (c = 1.027 in pyridine).

3.965 mg. substance: 3.360 mg. H_2O, 11.410 mg. CO_2.
3.367 " " : 2.860 " " 9.690 " "
$C_{33}H_{46}O_4$. Calculated. C 78.21, H 9.16.
Found. (a) " 78.48, " 9.48.
(b) " 78.49, " 9.53.

Tigogenone.—An acetic acid solution of 0.1 gm. of CrO_3 was added to a solution of 0.3 gm. of tigogenin in 10 cc. of acetic acid and the reaction mixture was heated on a steam bath for ½ hour. The solution was then diluted with 2 volumes of water and the product extracted with ether. The washed ether solution was evaporated to dryness and the residue was recrystallized from acetone. The substance separated in the form of plates which melted at 206–207° (204–205° according to Windaus (1)):

$[\alpha]_D^{24} = -35$ (c = 1.000 in pyridine).

4.810 mg. substance: 4.315 mg. H_2O, 13.785 mg. CO_2.
5.447 " " : 4.960 " " 15.620 " "
$C_{26}H_{40}O_3$. Calculated. C 77.94, H 10.07.
Found. (a) " 78.15, " 10.04.
(b) " 78.22, " 10.19.

Tigogenone Oxime.—A solution of 0.1 gm. of tigogenone in absolute alcohol was refluxed with an excess of hydroxylamine hydrochloride and sodium acetate for 3 hours. The alcohol was then removed and the residue was recrystallized from acetone. The oxime separated in the form of needles which melted at 256–258° with decomposition.

3.973 mg. substance: 3.595 mg. H_2O, 10.992 mg. CO_2.
4.212 " " : 3.855 " " 11.655 " "
5.000 " " : 0.142 cc. N (28°, 759 mm.)
$C_{26}H_{41}O_3N$. Calculated. C 75.13, H 9.95, N 3.37.
Found. (*a*) " 75.46, " 10.12.
(*b*) " 75.47, " 10.24.
(*c*) N 3.22.

Reduction of Tigogenone by Clemmensen's Method.—A solution of 0.1 gm. of tigogenone in 10 cc. of acetic acid was refluxed with 4 gm. of amalgamated zinc and 2 cc. of HCl (1.19) for ½ hour. The solution was diluted with 3 volumes of water and extracted with ether. The residue from this extract was crystallized from acetone. The product was recrystallized from methyl alcohol from which it separated as leaflets which melted at 265–267°. The yield was very poor.

4.050 mg. substance: 4.095 mg. H_2O, 12.065 mg. CO_2.
1.283 " " : 1.252 " " 3.780 " "
$C_{26}H_{42}O_2$. Calculated. C 80.76, H 10.96.
Found. (*a*) " 81.24, " 11.30.
(*b*) " 80.35, " 10.92.

Sarsapogenone.—0.3 gm. of sarsapogenin, which had been prepared (2) from commercial Honduras sarsaparilla root, was dissolved in 10 cc. of acetic acid. This was oxidized according to the procedure used in the case of tigogenin. The product was finally recrystallized from acetone from which it separated in the form of shining plates which melted at 220–222°.

$[\alpha]_D^{25} = -46$ (c = 1.027 in pyridine).

4.334 mg. substance: 4.020 mg. H_2O, 12.438 mg. CO_2.
3.956 " " : 3.670 " " 11.345 " "
$C_{26}H_{40}O_3$. Calculated. C 77.94, H 10.07.
Found. (*a*) " 78.27, " 10.38.
(*b*) " 78.20, " 10.37.

Sarsapogenone Oxime.—The oxime was prepared as se of tigogenone. The product was recrystallized from a m which it separated in the form of plates which melted 3°.

3.967 mg. substance: 3.590 mg. H_2O, 10.992 mg. CO_2.
4.716 " " : 4.260 " " 13.065 " "
5.410 " " : 0.155 cc. N (27°, 759.5 mm.).
$C_{26}H_{41}O_3N$. Calculated. C 75.13, H 9.95, N 3.37.
Found. (*a*) " 75.55, " 10.12.
(*b*) " 75.56, " 10.11.
(*c*) N 3.26.

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STROPHANTHIN

XVIII. ALLOCYMARIN AND ALLOSTROPHANTHIDIN. AN ENZYMATIC ISOMERIZATION OF CYMARIN AND STROPHANTHIDIN

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In a previous communication (1), the strophanthin which occurs in the seeds of *Strophanthus kombe* has been shown to be a mixture of glucosides of the aglucone strophanthidin. In all of these glucosides, strophanthidin is combined directly with cymarose, a methyl ether α-desoxy sugar, as cymarin. This glucoside was found to occur as such in small amount in the mixture but the major portion of the active glucoside was shown to consist of a further combination of cymarin with 1 or more molecules of glucose. By the action upon this mixture of an enzyme which was extracted from strophanthus seeds, it was found possible to hydrolyze all of the glucose linkages without affecting the union between cymarose and strophanthidin, with the result that the complex mixture of glucosides could be converted completely into cymarin. More recently we have attempted to make use of this fact in the course of the isolation from the seeds of the strophanthidin used in our structural studies. It was hoped that by passing through the stage of cymarin it would be possible to accumulate sufficient of the rare sugar, cymarose, for certain future studies. Instead, however, of employing the method already published, which consisted of the preliminary isolation of the crude, amorphous strophanthin mixture and digesting it with a crude enzyme which had also been isolated, a simplification of the process was attempted as follows: The ground and defatted seeds were mixed to a paste with water and after the addition of toluene the mixture was allowed to stand with the hope that the enzyme already present in the seeds would act directly upon the accompanying glucosides. In an examination of the resulting material, observations of a surprizing nature were made. Although the desired enzymatic cleavage of glucose from the complex strophanthins with the production of a simpler glucoside was found to occur, an additional apparently enzymatic transformation was noted, which we believe to be quite unique in character. The extent of this transformation depended upon the duration of the enzyme action. Following the usual procedure, the dilute alcoholic extract of the self-digested seeds after purification with lead acetate and concentration yielded a crystalline substance which proved to be not the expected cymarin. Contrary to cymarin, the new substance was sparingly soluble in chloroform and very soluble in methyl alcohol from which cymarin crystallizes so readily as a methylate. From the analytical results the formula $C_{30}H_{44}O_9$ was derived, so that the new glucoside is isomeric with cymarin. This conclusion was substantiated by the investigation of its hydrolytic products.

The isomerization of cymarin to this substance was found to have occurred in the aglucone portion of the molecule. On hydrolysis the same sugar was obtained which is contained in cymarin, namely cymarose (2). Instead of strophanthidin, however, another substance was obtained, the isomeric character of which was confirmed by the analysis leading to the formula $C_{23}H_{32}O_6$. Contrary to strophanthidin, it was readily obtained from solution in anhydrous form which melted at 248°. We have adopted the designation *allostrophanthidin* for this substance and for the parent glucoside that of *allocymarin*.

Investigation of allostrophanthidin has shown that the oxygen atoms contained in it have retained the functions possessed by those of strophanthidin. Allostrophanthidin still yields an *oxime*. On heating with alcoholic hydrogen chloride it behaves like strophanthidin (3) and is converted into the *ethyl half acetal of oxidodianhydroallostrophanthidin*, which is isomeric with the analogous derivative of strophanthidin. From these facts the aldehyde group is retained in this substance as well as the two tertiary hydroxyl groups which are removed as water to form the dianhydro derivative, and finally the secondary hydroxyl which participates in the oxidic linkage in the above half acetal. As in strophanthidin, the remaining 2 oxygen atoms belong to the lactone group. Allostrophanthidin still gives the nitroprusside reaction characteristic of the $\Delta^{\beta,\gamma}$-unsaturated lactones. In agreement with this, *dihydroallostrophanthidin* was obtained on hydrogenation. The attempt was made to prepare the usual iso derivative with methyl alcoholic alkali. A number of different procedures were tried and although a reaction was found to occur in each case no tangible crystalline substance could be isolated and the major reaction product proved to be always of amorphous acid character.

Since the transformation of strophanthidin into the new isomer has not involved a change of function of the characteristic groups of the former, this isomerization appears to be of purely stereochemical character. The above failure to obtain an isoallostrophanthidin with methyl alcoholic alkali suggests that a *cis-trans* reallocation has occurred of the groups participating in this reaction or of groups which may influence the reactivity of such participating groups. It was hoped that such an explanation might be checked by passing to trianhydroallostrophanthidin which might prove identical with trianhydrostrophanthidin, a benzenoid derivative. For this purpose, the ethylal of dianhydroallostrophanthidin was treated with concentrated hydrochloric acid. Instead, however, of the formation of the trianhydro derivative such as is produced in the case of strophanthidin, a chloro derivative was obtained. Since difficulty was experienced in obtaining the expected analytical figures with this substance it was not further investigated. For the present the exact relationship of allostrophanthidin to the parent strophanthidin remains undetermined.

The above transformation is apparently of enzymatic character. If the paste of kombe seeds was first heated to destroy the enzymes and was then allowed to stand, on working up the mixture only strophanthidin could be obtained on hydrolysis of the crude glucosides. In a former communication a crude enzyme, "strophanthobiase," was described (1), which had been employed for the cleavage of all of the glucosides into cymarin. At that time only cymarin was noted and no suspicion was obtained of the formation of any such substance as allocymarin. More recent experiments have confirmed these results. When a crude enzyme was prepared from kombe seeds in the usual manner and was precipitated by alcohol or acetone, such an enzyme when allowed to act [illegible] time on the crude glucoside mixture caused cleavage to cymarin only and no appreciable formation of allocymarin was noted. Either the effective isomerizing enzyme is not extracted by the method employed or it is very labile and easily inactivated by acetone or alcohol. Although it is probable that a proper procedure may be found for the separation of such an enzyme from the seeds, we have not for the present made further attempts in this direction. If the above conclusion is correct that we are dealing with a stereochemical rearrangement produced by an enzyme, the observation is of considerable interest. The only previous reference which we have been able to find concerning a stereochemical enzymatic transformation is the so called "Waldenase" of Fränkel (4). To such an enzyme this worker has referred the transformation of the usual *l*-tyrosine formed on tryptic digestion of casein to *d*-tyrosine through *d*-tyrosine anhydride on the prolonged action of trypsin.

Before the above work was begun we had accumulated in the course of the preparation of strophanthidin the cymarin which occurs as such in *Strophanthus kombe* seeds. This was secured as previously described by the extraction with chloroform of the purified alcohol-free solution of the crude glucosides and precipitation of this chloroform solution of crude cymarin with ligroin. Cymarin was then obtained as the methylate by recrystallization from methyl alcohol. In the final methyl alcoholic mother liquors considerable material remained and the attempt was made to repeat the chloroform extraction of this material for purification. A relatively small portion of a crystalline substance appeared at this point, which was very sparingly soluble in chloroform. After our experience with allocymarin we turned again to an investigation of this substance and found it to be identical with allocymarin. The latter, therefore, occurs as such in very small amounts in the seeds, a fact which is not at all strange in view of the artificial transformation described above. A small amount of such a transformation can quite conceivably occur in the seeds themselves.

An investigation of the pharmacological action of the new glucoside has also led to a surprizing result. It has been found to be practically devoid of the characteristic digitalis action in amounts which could be satisfactorily injected into frogs. The latter were found to tolerate as much as 2 mg. in a 35 gm. frog. Unfortunately the sparing solubility of the substance made it difficult to reach the toxic limit of the substance. 4 mg. of the substance in suspension in dilute alcohol failed to kill a 25 gm. frog. For such an animal 0.015 mg. of cymarin sufficed. If the chemical difference between cymarin and allocymarin is merely stereochemical, this difference in pharmacological action is of considerable interest.

Finally, in hydrogenation experiments with allostrophanthidin with the platinum oxide catalyst of Adams and Shriner, it was found that the reaction may be carried beyond the 1 mol stage which gives rise to the dihydro derivative described above. A second mol of hydrogen is more slowly absorbed with the formation of a neutral substance, *dihydroallostrophanthidol.* The second reaction was found to consist in the reduction of the aldehyde group to the primary alcoholic group. Since in our former experience with the catalytic hydrogenation of strophanthidin itself the platinum oxide catalyst had not been employed and such a reaction had not been noted, we have investigated the behavior of strophanthidin towards this reagent. 1 mol of hydrogen was fairly readily absorbed but apparently more slowly than in the case of allostrophanthidin and under very careful conditions it was found possible to introduce an additional mol of hydrogen with the formation of the dihydro alcohol, *dihydrostrophanthidol.*

EXPERIMENTAL

1500 gm. of *Strophanthus kombe* seeds which gave uniformly the green color with 80 per cent sulfuric acid were coarsely ground in a meat grinder and were defatted with gasoline. The dried powder was mixed with 3500 cc. of water and 100 cc. of toluene and the mixture was allowed to stand at 25° for 13 days. The mass was then treated with 12 liters of 95 per cent alcohol. After standing 24 hours the mixture was filtered. The filtrate was cleared as usual with lead acetate solution and the excess lead was removed with H_2S. During the concentration under diminished pressure in order to remove the alcohol, a copious crystallization occurred. At this point it was found advisable to centrifuge the precipitate and continue the concentration of the mother liquor until the alcohol was completely removed. The remaining aqueous solution was recombined with the crystalline fraction and the mixture was shaken with 200 cc. of chloroform to dissolve any cymarin which had escaped transformation. After centrifuging in order to separate the chloroform layer, the extraction with fresh chloroform was repeated. From the combined chloroform extracts, after shaking out with dilute sodium carbonate solution, concentration and precipitation with petrolic ether, 17 gm. of crude cymarin were recovered. This readily crystallized from methyl alcohol.

Allocymarin.—The above crystalline residue undissolved by the chloroform was collected by suction. After recrystallization from 95 per cent alcohol the glucoside formed globules of minute crystals which after collecting with alcohol and drying weighed 33 gm. When recrystallized by careful dilution of the hot alcoholic solution it formed long, narrow, thin leaflets which softened above 145° and slowly effervesced at about 150°.

Allocymarin is easily soluble in methyl alcohol and acetone, is less readily soluble in ethyl alcohol and practically insoluble in water. Contrary to the extremely bitter cymarin, it is practically tasteless.

$[\alpha]^{20}_{D} = +43$ (c = 1.007 in methyl alcohol).

For analysis the substance was dried at 100° and 15 mm.

4.523 mg. air-dry substance: 0.265 mg. H_2O.
$C_{30}H_{44}O_9 \cdot 2H_2O$. Calculated. H_2O 6.16. Found. H_2O 5.85.
4.258 mg. substance: 3.140 mg. H_2O, 10.210 mg. CO_2.
5.690 " " : 2.125 " AgI.
$C_{30}H_{44}O_9$. Calculated. C 65.66, H 8.09, OCH_3 5.65.
Found. " 65.39, " 8.25, " 4.93.

Allocymarin was obtained also by direct alcoholic extraction of the seeds without previous digestion. This was found in the mother liquors which had accumulated from the recrystallization from methyl alcohol of the cymarin fraction of the crude glucoside mixture. From about 200 pounds of such seeds about 15 gm. of allocymarin were obtained. It separated from 95 per cent alcohol in characteristic narrow, thin platelets which melted with slow effervescence at 150° after preliminary softening and agreed in all properties with the allocymarin prepared by digestion of the seeds.

$[\alpha]^{20}_{D} = +42$ (c = 0.970 in methyl alcohol).

The identity was confirmed by hydrolysis to allostrophanthidin and cymarose.

5.098 mg. air-dry substance: 0.323 mg. H_2O.
$C_{30}H_{44}O_9 \cdot 2H_2O$. Calculated. H_2O 6.16. Found. H_2O 6.33.
4.775 mg. substance: 3.520 mg. H_2O, 11.492 mg. CO_2.
$C_{30}H_{44}O_9$. Calculated. C 65.66, H 8.09.
Found. " 65.63, " 8.25.

The following experiment was made in order to obtain more conclusive evidence of the enzymatic origin of the transformation of the strophanthin glucosides into allocymarin.

500 gm. of kombe seeds after grinding and defatting yielded 350 gm. of dry powder. Separate portions of this material were treated differently as follows:

150 gm. of the seed powder were extracted three times with 700 cc. portions of 70 per cent alcohol. The combined extract was purified in the usual way with lead acetate and after removal of excess lead with H_2S the alcohol was removed under diminished pressure. The concentrated aqueous solution was cautiously acidified with HCl until slightly acid to Congo red paper. The mixture was then hydrolyzed at 70°. Characteristic rhombs and compact prisms of strophanthidin separated. After filtration a second crop was obtained from the filtrate by the addition of ammonium sulfate. After recrystallization each of these fractions melted at 170° and exhibited all of the properties of strophanthidin. The combined weight was 4.9 gm.

150 gm. of the seed powder were mixed with 450 cc. of water and 50 cc. of toluene and after digesting at 25° for 11 days the mixture was treated with 1400 cc. of alcohol. The alcoholic extract was worked up in the usual manner. In this case on concentration of the purified extract the glucoside crystallized as described above. The acidified aqueous suspension was heated at 70°. The sparingly soluble glucoside slowly dissolved and was soon replaced by long prisms and tables of allostrophanthidin. After recrystallization from alcohol 4.2 gm. of crystals melting at 247° were obtained. A second fraction which was recovered from the aqueous mother liquor of the first fraction by salting out with ammonium sulfate gave after recrystallization 1.6 gm. which melted at 240°.

A parallel experiment was conducted after destruction of enzymes as follows: 50 gm. of the seed powder were treated with 170 cc. of boiling water and the mixture was kept above 90° for 10 minutes. After cooling, 30 cc. of toluene were added and the mixture was kept alongside of the above experiment at 25° for 11 days. On hydrolysis of the resulting glucoside mixture only strophanthidin was obtained.

Hydrolysis of Allocymarin

Allostrophanthidin.—5 gm. of allocymarin were treated with a mixture of 35 cc. of alcohol, 25 cc. of H_2O, and 12 cc. of HCl (1.19). The resulting suspension was shaken at 20–25° for 24 hours. This time was required for solution and hydrolysis of the sparingly soluble glucoside. After dilution allostrophanthidin separated very incompletely. On partial concentration of the mother liquor to remove the alcohol most of the dissolved aglucone was recovered, although a small portion still remained in the aqueous sugar solution. This was recovered in the process of isolating the sugar as given below.

On recrystallization from 95 per cent alcohol allostrophanthidin separates as rather sparingly soluble prisms or plates which are anhydrous and melt at 248–250°, with slow effervescence. This melting point was found with the freshly recrystallized substance. In some cases after months of standing in the dry state the melting point was found to increase, and in one case 274° was noted. The substance is practically tasteless. It is sparingly soluble in the usual neutral solvents. It is more readily soluble in the somewhat diluted water-miscible solvents. It gives the characteristic nitroprusside test. The brown color which develops in sulfuric acid is indistinguishable from that produced by strophanthidin.

$[\alpha]_D^{20} = +37$ (c = 0.508 in 95 per cent alcohol).

3.815 mg. substance: 2.815 mg. H_2O, 9.577 mg. CO_2.

$C_{23}H_{32}O_6$.	Calculated.	C 68.27, H 7.98.
	Found.	" 68.45, " 8.25.

Cymarose.—The above aqueous filtrate from allostrophanthidin was treated with excess silver carbonate. The filtrate from silver chloride was treated with H_2S and then concentrated to small bulk under diminished pressure. A small additional deposition of the aglucone occurred during this process. The filtrate was then concentrated to dryness. The residue was extracted with dry ether and the extract was allowed to stand over fused Na_2SO_4 for several days. On being treated with petrolic ether, the concentrated ether solution gradually crystallized, when seeded with cymarose, as delicate needles. After recrystallization and long drying in a desiccator over $CaCl_2$ the sugar melted at 93°. This melting point, however, was obtained only with carefully dried material. When first collected or on exposure to the air small amounts of solvent or moisture which cling to the substance easily depress its melting point.

The sugar exhibited only slight mutarotation in aqueous solution. For the final reading

$[\alpha]_D^{27} = +52$ (c = 2.01 in H_2O).

3.610 mg. substance: 2.840 mg. H_2O, 6.880 mg. CO_2.
$C_7H_{14}O_4$. Calculated. C 51.81, H 8.70.
Found. " 51.98, " 8.80.

Allostrophanthidin Oxime.—This was prepared in the usual manner from the aglucone and hydroxylamine in alcoholic solution. It formed small four-sided tables and prisms from alcohol which effervesced at 235° after preliminary softening. For the analysis it was found necessary to dry the substance in the micro dryer at 120°.

4.536 mg. substance: 3.285 mg. H_2O, 10.925 mg. CO_2.
5.100 " " : 0.158 cc. N (772.6 mm., 28°).
$C_{23}H_{33}O_6N$. Calculated. C 65.83, H 7.93, N 3.34.
Found. " 65.69, " 8.09.
" N 3.57.

Dihydroallostrophanthidin.—Allostrophanthidin was hydrogenated in alcoholic solution with the platinum oxide catalyst of Adams and Shriner. Within 20 minutes approximately the equivalent of 1 mol of H_2 was absorbed and the operation was interrupted at this point. The dihydro derivative crystallized from dilute alcohol as long, narrow platelets which softened at 150° as if giving off solvent and finally melted and effervesced at 230°. It is easily soluble in alcohol and acetone and but sparingly so in chloroform. It does not give the nitroprusside reaction.

For analysis the substance was dried at 100° and 15 mm. over H_2SO_4.

4.365 mg. substance: 3.370 mg. H_2O, 10.920 mg. CO_2.
$C_{23}H_{34}O_6$. Calculated. C 67.99, H 8.44.
Found. " 68.22, " 8.64.

Dihydroallostrophanthidol.—When the above hydrogenation was carried further, an additional mol of H_2 was absorbed more gradually. The reaction was interrupted after 18 hours. The dihydro alcohol separated from its concentrated alcoholic solution as lustrous leaflets which melted at 222° after preliminary softening. The substance did not react with hydroxylamine.

For analysis the substance was dried at 100° and 15 mm. over H_2SO_4.

4.603 mg. substance: 3.650 mg. H_2O, 11.465 mg. CO_2.
$C_{23}H_{36}O_6$. Calculated. C 67.60, H 8.89.
Found. " 67.92, " 8.88.

Ethyl Half Acetal of Oxidodianhydroallostrophanthidin.—Allostrophanthidin was refluxed for 30 minutes in 10 parts of 5 per cent absolute alcoholic hydrochloric acid. On cooling needles of the ethylal separated.

After recrystallization from alcohol it melted at 191°.

$[\alpha]_D^{25} = -63$ (c = 1.01 in chloroform).

4.280 mg. substance: 3.135 mg. H_2O, 11.870 mg. CO_2.
4.302 " " : 2.430 " AgI.
$C_{25}H_{32}O_4$. Calculated. C 75.71, H 8.14, OC_2H_5 11.35.
Found. " 75.64, " 8.20.
" OC_2H_5 10.82.

Dihydrostrophanthidol.—Strophanthidin which had been repeatedly recrystallized from glass-distilled alcohol was hydrogenated as described in the case of allostrophanthidin. Within 12 minutes 1 mol of H_2 was absorbed. When interrupted at this point dihydrostrophanthidin was obtained. After 18 hours an additional equivalent of H_2 was absorbed. The filtrate from the catalyst crystallized on concentration and dilution. It formed needles and prisms from 95 per cent alcohol which melted slowly at 160–163° after preliminary sintering. Its melting point was definitely depressed on mixing with dihydrostrophanthidin and it is also less soluble in alcohol than the latter.

4.640 mg. substance: 3.760 mg. H_2O, 11.500 mg. CO_2.

$C_{23}H_{36}O_6$. Calculated. C 67.60, H 8.89.
Found. " 67.60, " 9.07.

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THE DIGITALIS GLUCOSIDES

V. THE OXIDATION AND ISOMERIZATION OF GITOXIGENIN

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Certain abnormalities in the behavior of isogitoxigenin and its derivatives as contrasted with that of isodigitoxigenin and other "iso genins" have repeatedly appeared in our studies with these substances and have been discussed in previous reports (1, 2). The outstanding differences were the stability of the lactol form of isogitoxigeninic acid which results on saponification of isogitoxigenin, and the relative stability of the lactone group in isogitoxigenic acid which results from the oxidation of this lactol group in the former acid. In the work which has led to the conclusion that gitoxigenin is hydroxydigitoxigenin (2), it was shown that the remaining free hydroxyl group in isogitoxigenic acid can be removed to form the anhydro acid. This anhydro acid, in contrast with the saturated parent acid, could be easily saponified by 0.1 N alkali. In addition it was readily hydrogenated with simultaneous cleavage of the lactone group to the desoxy acid, digitoxanoldiacid. Since the removal of this hydroxyl group influenced so greatly the stability of the lactone group, the conclusion was reached that this hydroxyl group must be situated on a carbon atom either within or adjoining the lactone ring. At the time it was assumed that in the oxidic ring of isogitoxigenin the same tertiary hydroxyl was involved as that which forms the oxidic ring of isodigitoxigenin. More recent observations have shown that this last assumption is not true and that different hydroxyl groups participate in the cyclization in each of these iso compounds. In the case of isodigitoxigenin this has been definitely shown to be the tertiary hydroxyl group (3). In isogitoxigenin this group has now been found to be the additional hydroxyl by which gitoxigenin differs from digitoxigenin. This was made evident by the fact that whereas gitoxigenin forms a dibenzoate (4) and digitoxigenin forms a monobenzoate isogitoxigenin on acylation yields only a monoacyl derivative, *isogitoxigenin benzoate.* The additional acyl group in gitoxigenin dibenzoate must be situated on the extra hydroxyl group which is not present in digitoxigenin and forms the oxidic ring of isogitoxigenin. This conclusion was confirmed by the study of the oxidation products of these substances.

The formation of isogitoxigenon on oxidation of isogitoxigenin with chromic acid has already been described (5). The production of a monoketone is in agreement with the assumed presence of one secondary hydroxyl group. This has been noted also in the case of digitoxigenin and isodigitoxigenin (6). On reexamining the so called gitoxigenon which was briefly described in a previous communication (7), observations have been made which do not agree with its provisional characterization as a monoketone. The originally published analytical figures did not agree so well with the required formula, $C_{23}H_{32}O_5$, but rather with the alternative formula $C_{23}H_{30}O_5$, also presented. Recent work has shown that in the formation of this substance two hydroxyl groups have been oxidized to carbonyl so that it is a dioxo derivative, $C_{23}H_{30}O_5$. It is, therefore, obvious that the extra hydroxyl group of gitoxigenin is not tertiary but is either of secondary or primary nature. The fact that the above isogitoxigenin forms only a monoketone and not a dioxo derivative is in agreement with the acylation results in determining the identity of the hydroxyl group involved in the oxidic ring of isogitoxigenin.

A further study of this oxidation product of gitoxigenin has led to the surprizing result that it no longer possesses the unsaturated lactone group of gitoxigenin. It does not give the nitroprusside reaction. Digitoxigenon, which was examined for comparison, was found to give the expected color reaction, a result in agreement with the fact that it can be isomerized by alkali to isodigitoxigenon (6). The explanation of the behavior of the dioxo compound from gitoxigenin was found in the fact that it is an iso compound. The oxidation of the extra hydroxyl group of gitoxigenin to carbonyl has induced a simultaneous isomerization of the type found in isodigitoxigenin in which the double bond is replaced by an oxidic bridge with the remaining tertiary hydroxyl. This was confirmed by the fact that it was not possible to convert the dioxo compound into an anhydro derivative by the action of mineral acid. As presented further on, such dehydration may be produced in the dioxo derivative of dihydrogitoxigenin in which isomerization to an iso derivative is no longer possible. The oxidation product of gitoxigenin is, therefore, a derivative of a normal isogitoxigenin which is isomeric with the isogitoxigenin formed by the alkali isomerization of the aglucone.

Further study of this oxidation product has brought to light an additional complication. It is a mixture of two isomers when prepared by the method originally given in which the chromic acid solution employed contained sulfuric acid. This was caused by an isomerization of the primary product of the reaction under the influence of acid. When the oxidation was accomplished in acetic acid solution with aqueous chromic acid, the principal product was a neutral dioxo derivative which showed a specific rotation of $[\alpha] = +88$. When this substance was allowed to stand with dilute mineral acid isomerization occurred with the formation of a substance possessing a rotation of $[\alpha] = +166$.

In view of the direct relationship of these substances to isodigitoxigenon, the first of these isomers has been at least provisionally designated as α-(*oxoisodigitoxigenon*) and the latter as β-(*oxoisodigitoxigenon*). When the α compound was gently saponified with dilute alkali and then relactonized by gentle acidification with acetic acid, isomerization also occurred since the substance recovered proved to be the above β compound. In an attempt to recrystallize the oxidation product from methyl alcohol it was found to yield a more sparingly soluble substance of higher melting point. This substance proved to contain methoxyl. Under the conditions described in the experimental part, either isomer was found to yield the same substance which proved to be a dimethyl acetal. The methoxyl groups were stable to alkali but were readily removed by dilute acid. This at first suggested the possibility that the extra carbonyl group is of aldehydic character. But a parallel experiment with isogitoxigenon showed that this substance also yields a dimethyl acetal with analogous properties. This is then a property of the carbonyl group common to both substances and which is in turn formed from the secondary alcoholic group common to both gitoxigenin and digitoxigenin. Such ready formation of a ketone acetal by simple heating with methyl alcohol is unusual, since it is a property usually credited to reactive aldehyde groups.

In the hope of shedding additional light on the mechanism of the formation and the properties of these isomeric dioxo compounds, we have turned to an analogous study of the oxidation of dihydrogitoxigenin where any simultaneous oxidation to the above type of iso derivative would be excluded, and in which the tertiary hydroxyl group would be retained. By using a chromic acid solution without the addition of sulfuric acid, the dihydrogenin yielded both a neutral fraction and an acid fraction. The neutral product proved to be mostly a dicarbonyl derivative, $C_{23}H_{32}O_5$, which possessed a specific rotation of $[\alpha] = +89$, and which we have called *α-(oxodihydrodigitoxigenon)*. When this substance was treated with mineral acid it was readily converted into an anhydro derivative, *anhydro-β-(oxodihydrodigitoxigenon)*. There was probably simultaneous isomerization to the β series. The rotation of the latter was found to be $[\alpha] = +154$. The dehydration appeared to keep pace with the apparent isomerization so that it was not possible to intercept any intermediate β form before such conversion into its anhydro form had occurred. When the above α-oxidation product was gently saponified and then relactonized by gentle acidification with acetic acid, the substance which crystallized proved to be also an anhydro compound but apparently still different from the above anhydro derivative. It appears that still another type of isomerization has occurred under the influence of alkali. This second anhydro compound was formed also by saponification and relactonization of the above anhydro-β-(oxodihydrodigitoxigenon).

The lability of the tertiary hydroxyl group with consequent anhydro formation may be conditioned by a *trans-cis* rearrangement of the hydrogen atom and hydroxyl group which are removed as water. The additional type of isomerization under the influence of alkali may be due to a further shift of the double bond. The loss of water during saponification is apparently caused by the influence of the extra carbonyl group. This was indicated by our experience with dihydrodigitoxigenin which was run as a control. This substance was readily converted into *dihydrodigitoxigenon.* When the latter was saponified and then gently acidified with acetic acid, as in the case of the gitoxigenin derivative, α-(oxodihydrodigitoxigenon), dehydration with the formation of an anhydro derivative did not occur and dihydrodigitoxigenon was recovered.

Another substance of interest was isolated from the acid fraction of the oxidation mixture obtained from dihydrogitoxigenin. This crystalline acid proved to be a constant by-product of the action of CrO_3 in the presence or absence of sulfuric acid and under different temperature conditions. From the analytical results the formula $C_{23}H_{32}O_6$ was derived for this substance. Titration showed the presence in it of one carboxyl group as well as its molecular size. This was confirmed by the analysis of the *methyl ester*. However, on heating even with 4 per cent alkali at 130° it was not possible to detect the consumption of the extra equivalent of alkali required by the lactone group originally present in the parent substance. Repeated experiments on attempted saponification with 0.1 N or N alkali gave the same result. On back titration in every case, however, it was found that about 10 to 15 per cent of 1 equivalent of alkali was nevertheless consumed in addition to that required for direct neutralization of the free acid group. A reason for this is difficult to picture and we are convinced of the homogeneity of this acid oxidation product. Contrary to the neutral dioxo derivative, the acid could not be converted into an anhydro acid by solution in concentrated hydrochloric acid. The neutral dioxo derivative is not an intermediate stage in the formation of this acid since the latter was not produced in an attempted reoxidation of the former.

The difficulty of procuring the rare and costly material needed in this work has made it necessary for the moment to interrupt further investigation of these substances in directions which are naturally suggested. The behavior of gitoxigenin and its dihydro derivative on oxidation, which has just been presented, has definitely shown that the additional hydroxyl is not of tertiary character as originally assumed and that it is situated on a carbon atom which is most likely δ to the γ-carbon atom of the lactone group of gitoxigenin.

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for the digitoxin-insoluble by-product which was the principal source of the starting materials used in these studies.

EXPERIMENTAL

Oxidation of Gitoxigenin

α-Dioxo Derivative (α-(Oxoisodigitoxigenon)).—The procedure used for the oxidation with chromic acid differs somewhat from that previously given (7).

A solution of 0.2 gm. of gitoxigenin in a mixture of 4 cc. of acetic acid and 1 cc. of water was treated with 1 cc. of 20 per cent aqueous CrO_3, a decided excess of reagent. After 15 minutes at 25° the diluted mixture was extracted with chloroform. The latter after washing with water and finally with dilute Na_2CO_3 was dried and concentrated. The residue crystallized under acetone and was collected with a few drops of acetone followed by acetone-ether (1:1). For recrystallization the concentrated acetone solution was carefully treated with dry ether. The α compound formed rhombic plates and prisms which melted at 200° after preliminary softening. When recrystallized from acetone alone, in which the substance is easily soluble, the melting point was raised to 204°. Contrary to gitoxigenin the dioxo derivative no longer gives the nitroprusside reaction.

$[\alpha]_D^{25} = +88$ (c = 0.386 in acetone).

4.460 mg. substance: 3.095 mg. H_2O, 11.660 mg. CO_2.

$C_{23}H_{30}O_5$.	Calculated.	C 71.46,	H 7.83.
	Found.	" 71.30,	" 7.77.

An appreciable amount of acid material which resulted during the oxidation was recovered from the carbonate extract but proved to be amorphous and resisted successful inquiry.

The α compound is isomerized to the β compound on treatment with dilute mineral acid or on saponification with NaOH and subsequent reacidification with acetic acid.

If, as originally published, the CrO_3 solution contains H_2SO_4 the substance resulting from the oxidation of gitoxigenin proved to be principally the β compound with smaller amounts of the α-isomer. The amount of the latter increased if the oxidation was carried on at low temperature and for a shorter time.

In one experiment 4 gm. of gitoxigenin were dissolved in 80 cc. of acetic acid and 20 cc. of water. The solution was treated with 20 cc. of Kiliani CrO_3 solution[1] and was allowed to stand at 5° for 7 minutes. When worked up as given above the product was found to be a mixture. The neutral residue from the chloroform extract was dissolved in acetone and carefully treated with dry ether. The crystalline substance was collected with ether. 2.9 gm. were obtained. When this was recrystallized by concentration of its acetone solution characteristic rhombic platelets of the α compound were obtained which after collection weighed 0.6 gm. This possessed all of the properties of the α derivative.

The material in the mother liquor proved to be a mixture from which on fractional crystallization by careful dilution of the acetone solution the β-isomer was obtained. This was, however, a tedious process. The β-isomer greatly preponderated and was more readily obtained if the oxidation was carried on at higher temperature and for a longer time as follows:

β-Dioxo Derivative (β-(Oxoisodigitoxigenon)).—The above reactants were kept at 27° for 1 hour and the resulting mixture was then worked up as usual. Careful addition of dry ether to the concentrated chloroform solution caused the gradual separation of needles which were collected with ether. After recrystallization from chloroform-ether the substance contained ether of crystallization and melted and slowly frothed up at 127–130°.

From dilute acetone the substance was obtained in anhydrous form and melted with effervescence at 213°.

$[\alpha]_D^{20} = +154$ (c = 0.676 in acetone).

For analysis the substance from chloroform-ether was dried first at 85° and finally at 105° and 15 mm.

4.365 mg. substance: 3.175 mg. H_2O, 11.400 mg. CO_2.

$C_{23}H_{30}O_5$.	Calculated.	C 71.46, H 7.83.
	Found.	" 71.16, " 8.13.

Like the α compound the β-isomer does not give the nitroprusside reaction. The failure to obtain an anhydro derivative by the action of acid under conditions which at once transformed gitoxigenin into the dianhydro derivative supports the interpretation that these oxidation products of gitoxigenin are isogitoxigenin derivatives in which the tertiary hydroxyl is bound in oxidic form. When the β compound was dissolved in HCl (1.19) and kept at 20° for 15 minutes the resulting solution on dilution gave an amorphous precipitate which could not be made to crystallize and which represented deep seated alterations. But from the clear filtrate a relatively small amount of crystalline material slowly deposited. After collection with water it was recrystallized by careful dilution of the acetone solution. It melted at 207–208° and proved to be starting material.

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[1] The solution consisted of 400 gm. of H_2O, 80 gm. of H_2SO_4, and 53 gm. of CrO_3.

$[\alpha]_D^{20} = +166$ (c = 0.217 in acetone).

4.020 mg. substance: 2.850 mg. H_2O, 10.477 mg. CO_2.
Found. C 71.08, H 7.93.

The β compound was also obtained directly from the α form by the action of dilute acid as follows: 0.1 gm. of α-(oxoisodigitoxigenon) was dissolved in a mixture of 2 cc. of acetic acid and 0.5 cc. of H_2O. The solution was then treated with 0.5 cc. of 25 per cent H_2SO_4. The change in rotation of the mixture kept at 20° was followed. From an initial direct reading in a 1 dm. tube of $\alpha = +2.18$, after 17 hours the reading had increased to +5.03. The reaction was then discontinued and the substance was extracted from the diluted mixture with chloroform. After recrystallization from dilute acetone it formed prisms which melted with effervescence at 213°.

$[\alpha]_D^{20} = +170$ (c = 0.507 in acetone).

4.600 mg. substance: 3.210 mg. H_2O, 12.050 mg. CO_2.
Found. C 71.45, H 7.81.

The α form was converted into the β-isomer by the action of alkali as follows: 0.1 gm. of the α compound dissolved in 2 cc. of acetone was treated with 2 cc. of 2 per cent NaOH. After solution had occurred the mixture was allowed to stand for 2 hours at 25°. After dilution and reacidification with acetic acid it slowly crystallized. The substance was collected with water and because of the small amount it was analyzed directly. It melted at 206°

and proved to be the β form since $[\alpha]_D^{20} = +166$ (c = 0.350 in acetone).

4.533 mg. substance: 3.210 mg. H_2O, 11.750 mg. CO_2.
Found. C 70.70, H 7.93.

Dimethyl Acetal of β-(Oxoisodigitoxigenon).—When the attempt was made to recrystallize either the α or β compounds from methyl alcohol, the resulting substance was found to contain methoxyl. The addition of methyl alcohol was, however, incomplete and a mixture resulted which melted higher than the starting material. The acetal was prepared as follows: 0.1 gm. of the β form was heated in 10 cc. of anhydrous methyl alcohol in a sealed tube at 100° for 18 hours. On rubbing, a copious crystallization gradually separated. After recrystallization from methyl alcohol in which the methylal is sparingly soluble, it formed flat needles and platelets which melted with effervescence at 226–227°.

4.392 mg. substance: 3.240 mg. H_2O, 11.200 mg. CO_2.
3.595 " " : 3.855 " AgI.
$C_{25}H_{36}O_6$. Calculated. C 69.40, H 8.39, OCH_3 14.34.
Found. " 69.55, " 8.25, " 14.15.

The above reaction was not complete and the material contained in the mother liquors was found to be low in methoxyl content.

After saponification of the acetal and careful reacidification with dilute acetic acid, a neutral substance was recovered which proved to be starting material. If the action of acid is prolonged, however, hydrolysis of the acetal group occurs with formation of the free ketone, as shown in the following experiment.

A solution of 0.1 gm. of the acetal in 20 cc. of warm acetone was treated with 20 cc. of water containing 1 cc. of acetic acid. The clear solution was left at room temperature for a day. On concentration the substance crystallized. After recrystallization from dilute acetone it melted with effervescence at 213° and proved to be methoxyl-free.

Dimethyl Acetal of Isogitoxigenon.—Isogitoxigenon (1) was heated with 50 parts of dry methyl alcohol at 100° for 20 hours. On concentration to small volume the mixture crystallized slowly and incompletely at 0°. As it separates from methyl alcohol the substance melts at 217° with effervescence. On recrystallization from acetone it forms leaflets which melt at 235°. Contrary to the acetal of β-(oxoisodigitoxigenon) this substance separated in almost pure form from the concentrated reaction mixture, leaving in solution a large proportion of more soluble partially methylated material.

4.208 mg. substance: 3.277 mg. H_2O, 10.655 mg. CO_2.

$C_{25}H_{38}O_6$.	Calculated.	C 69.08,	H 8.82,	OCH_3 14.27.
	Found.	" 69.06,	" 8.71,	" 13.58.

The acetal nature of this substance was shown by its instability towards acetic acid. 0.04 gm. was dissolved in 5 cc. of acetone and then treated with 5 cc. of 10 per cent acetic acid. The crystals of acetal which reprecipitated soon redissolved due to hydrolysis. On concentrating to remove acetone, a substance crystallized which melted at 280° and was methoxyl-free. It was, therefore, regenerated isogitoxigenon.

Oxidation of Dihydrogitoxigenin

α-Dioxodihydro Derivative (*α-(Oxodihydrodigitoxigenon)*).—2.8 gm. of dihydrogitoxigenin were dissolved in a mixture of 60 cc. of acetic acid and 15 cc. of water and then treated with 15 cc. of 20 per cent aqueous CrO_3. Oxidation occurred fairly promptly and after 40 minutes at 25–30° the mixture was diluted and extracted with chloroform. The chloroform extract was repeatedly washed with water to remove acetic acid and then finally with water to which just sufficient Na_2CO_3 was added to make it faintly alkaline to phenolphthalein. This process extracted the acid oxidation product which will be described below.

The dried chloroform solution of the neutral fraction on concentration left a resin which crystallized when treated with a small amount of acetone in which it is appreciably soluble. After collection with a small amount of chilled acetone it was recrystallized by careful addition of dry ether to the concentrated acetone solution. It formed platelets and prisms which softened to a paste at 200° and melted at 220–221°.

$[\alpha]_D^{20} = +89$ (c = 0.347 in acetone).

3.420 mg. substance: 2.550 mg. H_2O, 8.885 mg. CO_2.

$C_{23}H_{32}O_5$.	Calculated.	C 71.08,	H 8.31.
	Found.	" 70.86,	" 8.34.

This dioxo compound is isomerized with simultaneous loss of water under the influence of mineral acid or alkali to form the following anhydro derivatives.

Anhydro-β-(Oxodihydrodigitoxigenon).—0.08 gm. of the above dihydrodioxo compound was dissolved in 2 cc. of acetic acid and 1 cc. of 10 per cent HCl. The rotation was then followed in a 1 dm. tube and was found to increase from an initial $\alpha = +2.00$ to $+3.31$ after 22 hours. At this point it was diluted and extracted with chloroform. The washed chloroform extract left, on concentration, a resin which readily crystallized under acetone. On recrystallization from acetone in which it is quite soluble, it formed platelets and needles which melted at 240°. It was obtained in the same form also from acetone-ether.

$[\alpha]^{20}_{D} = +154$ (c = 0.28 in acetone).

4.165 mg. substance: 3.020 mg. H_2O, 11.380 mg. CO_2.

$C_{23}H_{30}O_4$.	Calculated.	C 74.54, H 8.17.
	Found.	" 74.52, " 8.11.

This substance was saponified by solution in a mixture of equal parts of 4 per cent NaOH and acetone and the solution was allowed to stand for several hours. After acidification the relactonized substance crystallized. On recrystallization from acetone by addition of ether it formed needles which melted at 219°. This melting point remained the same after several recrystallizations. This suggested that a further isomerization of the anhydro compound in addition to the α to β transformation occurs under these conditions.

The same isomeric substance was also obtained by the action of alkali directly on α-(oxodihydrodigitoxigenon) as follows: 0.1 gm. of the α-dihydrodioxo compound was treated with a mixture of 2 cc. of 2 per cent NaOH and 1 cc. of alcohol. Solution gradually occurred due to saponification of the lactone. After 1 hour at 25–30° the mixture was diluted and acidified gently with acetic acid. As relactonization occurred, the substance gradually crystallized. On recrystallization from acetone-ether it formed needles which melted at 220°.

$[\alpha]^{27}_{D} = +173$ (c = 0.359 in acetone).

3.957 mg. substance: 2.840 mg. H_2O, 10.772 mg. CO_2.

$C_{23}H_{30}O_4$.	Calculated.	C 74.54, H 8.17.
	Found.	" 74.25, " 8.03.

Acid, $C_{23}H_{32}O_6$.—The dilute Na_2CO_3 extract of the acid material obtained from the above oxidation mixture was concentrated under diminished pressure to a small volume. On gentle acidification with acetic acid a gum was precipitated. On being rubbed and left to stand this was followed by crystallization of micro rhombs and prisms with gradual crystallization of the gum. After 24 hours this was collected. The yield was 0.6 gm. An additional 0.3 gm. was obtained by the addition of saturated ammonium sulfate to the mother liquor. The acid was dissolved by the addition of a few drops of water to a suspension in warm acetone. After concentration it crystallized as compact prisms which melted at 246° with effervescence. The acid is sparingly soluble in dry acetone and chloroform and readily soluble in alcohol.

$[\alpha]_D^{25} = -74$ (c = 0.377 in acetone-water (4:1)).

For analysis and titration the substance was dried at 100° and 15 mm.

Sample a. 4.265 mg. substance: 3.053 mg. H_2O, 10.645 mg. CO_2.
" b. 4.374 " " : 3.105 " " 10.965 " "
$C_{23}H_{32}O_6$. Calculated. C 68.27, H 7.98.
Found. Sample a. " 68.07, " 8.01.
" b. " 68.35, " 7.94.

10.830 mg. of substance were treated with 1 cc. of alcohol and directly titrated with 0.1 N NaOH against phenolphthalein. Found, 0.261 cc. Calculated for 1 equivalent, 0.268 cc.

12.747 mg. of substance on direct titration required 0.317 cc. Calculated for 1 equivalent, 0.315 cc. 3.0 cc. of 0.1 N NaOH were then added and after being refluxed for 2 hours the mixture was titrated back. Found, 0.052 cc. or only 16.5 per cent of the theory for an extra equivalent.

92.480 mg. of substance were heated in a sealed tube with 1 cc. of alcohol and 4.877 cc. of N NaOH at 130° for 5½ hours and then titrated back. Found, 0.251 cc. Calculated for 1 equivalent, 0.229 cc. The presence of a lactone group, therefore, could not be directly determined.

In working up the acid fraction of this substance, dilute Na_2CO_3, which was definitely alkaline to phenolphthalein, was used and therefore a possibility appeared that saponification first occurred and then relactonization after acidification on another carboxyl. In order to obviate such a possibility dilute $NaHCO_3$ was used to extract the acid, the solution never reaching an alkalinity sufficient to turn phenolphthalein. The acid obtained in this experiment proved to be identical in all respects with the above acid.

In an attempt to detect the possible retention of the tertiary hydroxyl group in this acid, its behavior towards HCl was examined as follows: 0.1 gm. of the acid was dissolved in 2 cc. of HCl (1.19) at 0°. After 15 minutes the solution was diluted, which caused the separation of some amorphous material. The filtrate on treatment with ammonium sulfate solution deposited a crystalline substance which was recrystallized from dilute acetone. It melted at 247° and proved to be starting material.

$[\alpha]_D^{25} = -75$ (c = 0.36 in acetone-water (4:1)).

4.360 mg. substance: 3.250 mg. H_2O, 10.870 mg. CO_2.
Found. C 68.00, H 8.34.

With the idea that the neutral α-dioxo compound might be intermediate in the formation of this acid the attempt was made to reoxidize the former with CrO_3. No acid product was obtained and α-(oxodihydrodigitoxigenon) was recovered.

Methyl Ester of the Acid, $C_{23}H_{32}O_6$.—This was prepared from the acid with diazomethane in acetone solution. On recrystallization from dilute methyl alcohol it formed platelets which melted at 212°.

4.337 mg. substance: 3.230 mg. H_2O, 10.960 mg. CO_2.
3.772 " " : 2.140 " AgI.
$C_{24}H_{34}O_6$. Calculated. C 68.86, H 8.19, OCH_3 7.41.
Found. " 68.92, " 8.33, " 7.49.

Isogitoxigenin Benzoate.—Isogitoxigenin was benzoylated in pyridine solution with an excess of benzoyl chloride. The mixture was allowed to stand about 18 hours and then poured into dilute H_2SO_4. The benzoate readily crystallized from alcohol as needles which melted at 248°.

For analysis the substance was dried at 100° and 15 mm.

5.088 mg. substance: 3.520 mg. H_2O, 13.550 mg. CO_2.
$C_{30}H_{38}O_6$. Calculated. C 72.83, H 7.75.
Found. " 72.63, " 7.73.

Gitoxigenin Dibenzoate.—Gitoxigenin was benzoylated under conditions identical with those used in the case of isogitoxigenin. The substance proved to be a dibenzoate as already reported by Cloetta (4) and Windaus and Schwarte (4).

4.867 mg. substance: 3.125 mg. H_2O, 13.188 mg. CO_2.
$C_{37}H_{42}O_7$. Calculated. C 74.21, H 7.08.
Found. " 73.91, " 7.18.

Dihydrodigitoxigenon.—Dihydrodigitoxigenin was oxidized in 80 per cent acetic acid solution with aqueous CrO_3. After 15 minutes at 8° the diluted mixture was extracted with chloroform. After the extract was washed with water and dilute carbonate the ketone was readily obtained on concentration. It formed needles from dilute acetone which melted at 160–162°.

4.605 mg. substance: 3.750 mg. H_2O, 12.505 mg. CO_2.
$C_{23}H_{34}O_4$. Calculated. C 73.75, H 9.15.
Found. " 74.06, " 9.11.

The acid fraction which was extracted with dilute carbonate was negligible in amount.

The ketone was gently saponified by solution in 2 per cent NaOH to which a half volume of alcohol was added. After dilution and gentle acidification with acetic acid it slowly crystallized. This effervesced at 163° and proved to be starting material and not an anhydro derivative as in the case of the corresponding dihydrogitoxigenin derivative.

5.260 mg. substance: 4.420 mg. H_2O, 14.300 mg. CO_2.
Found. C 74.14, H 9.39.

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[Reprinted from JOURNAL OF THE SOCIETY OF MOTION PICTURE ENGINEERS, October, 1930, Vol. 15, No. 4, pp. 439-444]

PROGRESS IN MICRO CINEMATOGRAPHY

BY HEINZ ROSENBERGER*

(From The Rockefeller Institute for Medical Research)

At the fall meeting in 1927 a paper on *Micro Cinematography* was given in which the usefulness of the motion picture not only for the demonstration of microscopic phenomena but also its application in medical and biological research work was described.

The work done in the last two years has been toward improving the technic of taking micro cinema records and also toward the improvement and the standardization of the necessary equipment. At the spring meeting in 1929, a description of the Standard Micro Cinematographic Apparatus was given. With this apparatus every scientist is able to obtain good results as the handling of the apparatus is very much simplified. It is fully automatic and can be left going for days without attention excepting the control of focus from time to time and more often when higher magnifications are used.

For accelerated film records, and most of the cell work is accelerated, the frequency, for instance 1, 2, 3, 4, 6, 8, 12, *etc.*, exposures per minute can be adjusted on a dial of an electric clock. The length of exposure can be adjusted on a screw on the timing device, so also can the time be set when the machine should start or stop by the use of an additional time clock.

The number of adjustments on the standard apparatus is now reduced to a minimum and therefore the handling is much simplified, leaving the attention of the operator mainly on the subject to be photographed. When an object, for instance a living cell, is to be photographed the following manipulations have to be done: After placing the slide under the microscope the object can be viewed through the focusing device in very much the same way as by ordinary observation. The rate of exposure is then to be adjusted by turning a hand on the dial to the proper number. Then a test strip of film can be taken and developed. Under or over exposures can be regulated by turning the adjusting screw of the timing device either to the left for longer exposure or to the right for shorter exposure. For higher frequencies and when using arc light, the sector of the revolving shutter has to be adjusted.

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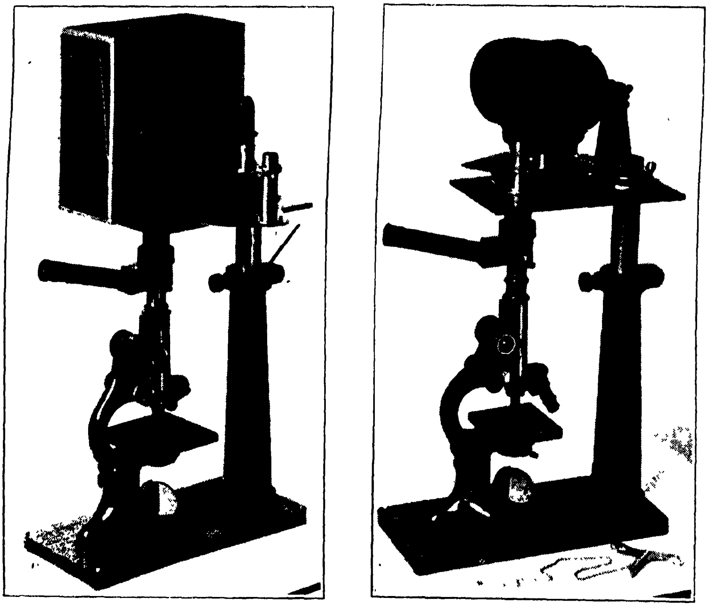
* Read before the Society at Washington.

The main direction of our research work during the last two years, since the micro film was shown, was toward higher magnifications in order that something about the structural details of living tissue cells may be revealed. It was also anticipated to keep cells alive and under observation for a longer period of time than was possible before. This was accomplished by the development of a special culture chamber, which was manufactured with the coöperation of Zeiss.



With Cine Kodak Model A With Filmo Camera

Micro cinema apparatus for 16 mm. cameras

I want to point out here that the difficulties of micro cinema work with high magnifications increase by arithmetical progression. Suppose we are looking at the moon through a telescope. When it is a low powered telescope the moon will move slowly across the field of vision, due to the rotation of the earth. With medium powered telescopes the moon apparently will move very much faster across the field, while with high powered telescopes one can hardly follow the motion.

The same is true with the microscope. The higher the magnification, apparently the faster is the speed with which the objects move. The faster the speed of the objects, the more frequent exposures we have to make. The more exposures we take per minute or second, the greater must be the light intensity to give the film the proper exposure, but the more injurious is the light to the delicate objects. And last but not least, the higher the power of the objective the less depth of focus we have in the field and the more difficult it is to keep the preparation in focus. I have not yet mentioned the great danger of transmitting vibrations from the moving parts of the apparatus or from some outside disturbances to the microscope, for naturally vibrations also are magnified.

Magnifications are measured with an object micrometer, in which one mm. is divided into 100 parts. This micrometer is photographed on the film.

The magnifications which were mainly used several years ago were ranging from 60 to 120 X on the film frame. Last year's experiments were generally made with a magnification of about 600 X on the film frame. In several cases, however, a magnification of 1250 X, which is about the limit, was used. It should be mentioned here that these figures should be multiplied by about 2.5 in order to obtain the magnifications with the same optical equipment when one looks through the microscope. The highest magnification would then be around 3000 X. Abbe claimed that the limit of microscopic magnification in white light is reached at about 1500 X.

Another point of consideration, especially when dealing with living cells under high magnifications, is the lack of contrast of these delicate structures. Very often these cells cannot be recognized by a layman at all when he looks through the microscope, as their appearance is clear as glass and there is very little difference between the cells and the surrounding medium. A vital stain, such as neutral red or Janus green, as frequently used in tissue culture work has not been used with high magnifications as these stains seem to have a deleterious effect on the cells in combination with the strong light. Because of some chemical reactions within the cells or else because of the absorption of heat, the cells either do not behave normally or else die very rapidly.

It is possible, however, to increase contrasts photographically. It is a well-known fact that various photographic emulsions can reproduce an object with greater contrast than actually exists. It is possible to photograph, for instance, an article with very slight variations of gray, hardly noticeable to the human eye, so that these variations become almost black and white in the reproduction. The photographic emulsion can detect differences in shade which are invisible under normal circumstances. We have employed the above-mentioned properties with great advantage in micro cinema work in the detection of cell structures which are otherwise invisible. Motion picture records have been made, for instance, of white blood cells which show clearly that these cells are surrounded by an undulating membrane, much larger in area than the cell itself. Furthermore, records have been taken of *fibroblast* cells in which the *mitochondria* move about like snakes. The function of these cell bodies is not as yet clearly understood.

A Micro Cinematographic Apparatus for 16 Mm. Motion Picture Cameras

In order to enable the owner of a 16 mm. motion picture camera to make film records of microscopic phenomena a small and inexpensive outfit has been designed which has many uses in a scientific laboratory, especially where objects have to be photographed without going through cumbersome preparations.

The outfit consists of the following parts: the base plate on which the microscope is placed, an adjustable column with two key ways and two set screws, a fixed plate with camera opening, a swivel plate, a camera holder, a combination focusing and beam centering device, and a telescope connecting sleeve. This apparatus adapted for the Bell & Howell Filmo and for the Eastman Cine Kodak Model A is illustrated in the figure.

The handling of the apparatus is very simple. The microscope, of any make, can always be used in the ordinary way and is only placed on the base plate when film records have to be taken. When brought in line with the focusing device once it will always be in line thereafter, when metal strips are screwed on the base plate after the first adjustment. Then the connecting sleeve is put in place. It should hang from the fixed plate into a microscope collar of cardboard or metal without touching it. The object can be viewed through the focusing tube in very much the same way as by ordinary microscopic observation, using coarse or fine adjustment on the microscope. Then the picture can be taken at once by swinging the camera in position over the microscope. The beam of light needs only to be adjusted once by looking into the beam centering tube and at the same time turning the microscope mirror into the proper position so that there is an even distribution of light over the field.

For general work there is practically no vibration transmitted from the camera to the microscope and many pictures have been taken even with very high magnifications, being absolutely sharp in focus. Sometimes, however, it may become necessary, especially when preparations in the "hanging drop" are to be taken, to safeguard them from the slightest amount of tremor. This is easily done by placing the microscope on a separate table and turning the entire upper part of the apparatus 180 degrees, so that the focusing tube is again in line with the microscope. The focusing and taking of films are otherwise done in the same manner as described above.

Any make of 16 mm. motion picture camera can be used with this outfit.

SYNOPSES OF TWO REELS

Demonstrating Motion Pictures of Microscopic Objects

Reel 1.—Living Cells of the Blood.
  The beating heart of a frog.
  The blood flow through arteries, veins, and capillaries.
  Various types of white blood cells.
  Macrophages from Jensen *sarcoma.*
  Sudden death of a *macrophage.*
  Cells of *necturus* showing thread-like *pseudopods.*
  Cells of *necturus,* high magnification, showing membrane.
  Records of cell divisions and *phagocytosis.*

Reel 2.—Cells of Living Tissue.

Beating fragment of heart tissue from a chick embryo.
Growing culture of *fibroblasts,* low power.
Two single *fibroblast* cells, high magnification, showing d ucture.
Normal rat *fibroblast.*
Malignant rat *fibroblast.*
Details of cell structures under very high magnification, nucleus, *nucleolus, mitochondria, etc.*
Rat *fibroblast* forming new cell branch.
Culture of nerve fibers.
Growing end of a single nerve fiber.
Pigment *epithelium,* high magnification.
Cell division of a *fibroblast* (high power).
Cell division of a *fibroblast* (very high power) showing interior of cell.

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[10] ROSENBERGER, HEINZ: "A Standard Micro Cinematographic Apparatus," *Trans. Soc. Mot. Pict. Eng.,* **XIII** (1929), No. 38, p. 461.

[11] ROSENBERGER, HEINZ: "A Micro Cinematographic Apparatus for the Owner of a 16 mm. Motion Picture Camera," *Science,* **LXXI** (1930), p. 266.

DISCUSSION

PRESIDENT CRABTREE: Gentlemen, I think we have witnessed one of the finest motion photomicrographs ever made. No doubt these researches of the photomicroscopist are going to throw a great deal of light on metabolism and the causes of health and disease.

Mr. Zworykin: Is this rapid motion of particles shown on the film similar to the Brownian movement, and is it the result of the bombardment of the larger particles by the random motion of the molecules with some oriented components?

Mr. Rosenberger: It may be that there is a flow of protoplasm inside of the cells. Those granules in the film are very large compared with the small ultramicroscopic particles, the motion of which is presumably caused by bombardment. I have taken a film of ultramicroscopic particles in motion (Brownian movement) of gold and silver colloids which I have here and which I can show if you wish. In order to shorten my paper I refrained from showing it.

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STUDIES ON PENETRATION OF DYES WITH GLASS ELECTRODE

IV. PENETRATION OF BRILLIANT CRESYL BLUE INTO NITELLA FLEXILIS

BY MARIAN IRWIN

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Accepted for publication, June 19, 1930)

I

INTRODUCTION

Although basic dyes play a special rôle in biology, because many of them are readily taken up by living cells, the form in which they penetrate has never been satisfactorily determined.

A basic dye is usually obtained in the form of the salt some of which may be transformed in aqueous solution to free base (the amount depending on the "apparent" dissociation constant of the dye and the pH value), and in the range of pH values generally employed with living cells (approximately between pH 5 and 9.5) both forms of dye must be considered. In this range methylene blue, for example, exists only as a salt. It is very strongly basic[1] so that even at pH 9.5 no free base is formed. Lauth's violet, on the other hand, is less basic, with a basic dissociation constant[1] of 1.9×10^{-3}.

Since the nature of the transformation of the dye salt into the free base is still in dispute[2] the structure of the salt and free base of the dye will not be discussed.

Furthermore, the dissociation constant of cresyl blue has not been determined by such methods as the oxidation-reduction potential or the glass electrode, so that the basicity of the dye is not accurately known.

It is, however, possible to determine approximately the relative ratio of the dye in form of free base/salt by shaking the chloroform with the dye solution and determining colorimetrically the "apparent" partition coefficient $k_{ap} = \frac{C_1}{C_2}$, where C_1 is the concentration of free base of the dye in chloroform and C_2 the concentration of the total dye (free base in equilibrium with the salt) in the aqueous solution. Such a determination was previously made[3] and it was found that the values of the k_{ap} increased with rise in the pH values as if the basic dissociation constant of the dye were about $10^{-5.6}$. But owing to the extreme solubility of the free base in chloroform and to the progressive alteration in the color of the dye in the aqueous solution from about pH 10 upward, it was not possible to determine the value of the true partition coefficient $k_{tr} = \frac{C_3}{C_4}$ where C_3 represents the concentration of free base of the dye in chloroform and C_4 the same in the aqueous solution. The pH value at which the dye existed almost entirely in form of free base was assumed and the value of k_{tr} was accordingly calculated. The dissociation constant was then calculated on basis of this assumption so that it may prove to be different when the determination is made with a more reliable method. But the significance of this observation does not lie in the accurate determination of the dissociation constant but in the proof that the relative increase in the values of k_{ap}, showing roughly the relative ratio of free base/salt, more or less corresponds to the relative increase in the rate of penetration of the dye into *Nitella* as the pH value of the aqueous dye solution is raised.

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[1] Clark, W. M., Cohen, B. C., and Gibbs, H. D., *Hygienic Laboratory Bulletin*, 1928, No. 151, 202-206.

[2] Henrich, F., Theories of organic chemistry, translated by John Johnson and Dorothy Hahn, London, 1922.

It has been suggested that basic dyes penetrate living cells in the form of free base[4] and not in the form of salt,[5] but this, despite many attempts, has never been really proven. The rate of penetration of a basic dye has been found to increase with a rise in the pH value of the external dye solution. This observation might be interpreted as showing penetration in the form of free base since the concentration of this form of dye increases as the external pH value rises. But it might be held to indicate that the dye penetrates as positive ions since with a rise in the external pH value an increase of the negative charges in the cell surface might occur which might result in greater penetration of the positive dye ions.

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[3] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, **23**, 251; *J. Gen. Physiol.*, 1925–26, **9**, 561.

[4] The following authors assume that basic dyes penetrate as free base. Overton, E., *Jahrb. wissensch. Bot.*, 1900, **43**, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, **10**, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, **4**, 1. McCutcheon, M., and Lucké, B., *J. Gen. Physiol.*, 1923–24, **6**, 501. Irwin, M., *J. Gen. Physiol.*, 1925–26, **9**, 561.

[5] The following authors account for the penetration of basic dyes through combining of dye cations with protoplasmic constituents. Bethe, A., *Biochem. Z.*, 1922, **137**, 18. Rohde, K., *Arch. ges. Physiol.*, 1920, **182**, 114. Pohle, E., *Deutsch. med. Wochn.*, 1921, **47**, 1464. Collander, R., *Jahrb. wissensch. Bot.*, 1921, **60**, 354.

If the dye penetrates as free base and combines[6] with the sap to form salt which annot readily escape from the vacuole, an increase in the pH value of the sap might e expected to decrease the rate of penetration. When the pH value of the sap of *Nitella* was increased by entrance of ammonia, the rate of penetration of cresyl blue was decreased[7] from which it was concluded[7] that the dye penetrated as free base. If such a result were indicative of the penetration of the dye as free base we should expect that in decreasing[8] the pH value of the sap an increase in the rate of penetration of cresyl blue would take place. But on lowering the pH value of the sap, through the entrance of acetic acid, a decrease[8] in the rate of penetration was observed, instead of an increase. This decrease may be due to an inhibitory effect of the acid on the protoplasm irrespective of the changes in the sap since various[9] other substances produce an inhibiting effect without penetrating the vacuole.

It is therefore desirable to find a satisfactory way of distinguishing between the penetration of the free base and that of the dye salt. This can be done by observing the effect of the dye on the pH value of the sap. The hydrogen electrode cannot be utilized, owing to the poisoning of the electrode by the dyes and to their oxidation-reduction potential, and the glass electrode is therefore employed. Since this requires the use of about 0.4 cc. of sap it is necessary to choose large cells which yield sap in sufficient amount for analysis. This can easily be collected from the vacuoles of cells of *Nitella*. Its behavior toward dyes is roughly comparable to that of such cells as *Paramecium*, *Spirogyra*, *Elodea*, and *Valonia* so that by studying the mechanism of dye penetration with *Nitella* we may gain some insight into the behavior of other cells as well.

In the present paper therefore determinations of the pH values of the sap of *Nitella* by the glass electrode[10, 11] are used as a means of studying the form of dye penetrating the vacuoles of living cells.

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[6] E. N. Harvey was the first to suggest that the free base was converted to the salt in the sap. See reference under footnote 4.

[7] McCutcheon, M., and Lucké, B., *J. Gen. Physiol.*, 1923–24, **6**, 501.

[8] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, **24**, 54; *J. Gen. Physiol.*, 1926–27, **10**, 271; 1927–28, **11**, 111.

[9] See footnote 8, and also Irwin, M., *J. Gen. Physiol.*, 1926–27, **10**, 425.

[10] Through the kindness of Dr. D. A. MacInnes and of Dr. M. Dole a glass electrode apparatus was devised with which it was possible to measure a very small amount of liquid. Through their cooperation it was possible to determine whether this method was applicable to the studies of the penetration of cresyl blue into *Nitella* cells, as mentioned in their article (MacInnes, D. A., and Dole, M., *J. Gen. Physiol.*, 1928–29, **12**, 805).

[11] Applicability of the glass electrode methods to the measurement of the sap, mentioned under footnote 9, was described separately by Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1929–30, **27**, 132. Preliminary report of the present paper was published, *Proc. Soc. Exp. Biol. and Med.*, 1929–30, **27**, 991.

II

Methods

Solutions Employed.—All buffer solutions were made up according to Clark's[12] standards and diluted ten times. The pH value of these solutions was checked by means of the glass electrode. The dyes were in all cases dissolved in these dilute solutions.

Condition of the Dye Used.—The cresyl blue was purchased as a salt. The brilliant cresyl blue (called cresyl blue in the text) was manufactured by Grübler previous to 1914, and appears to contain (in the range of pH values between pH 5.5 and pH 9.2) chiefly cresyl blue in form of salt or free base. It also contains another dye, X, but in the solutions employed the amount is so small in comparison to the cresyl blue (free base or salt) that X may be neglected. The difference in behavior between the cresyl blue and X is well marked so that it can be readily distinguished. Cresyl blue is absorbed as free base by the chloroform and appears orange. This orange dye can be entirely removed from the chloroform on shaking the chloroform with the artificial sap of *Nitella* (0.1 N KCl at pH 5.5) in which it appears blue. On shaking the cresyl blue solution with chloroform, the dye is absorbed much more from pH 9.2 than from pH 5.5, showing that the free base of cresyl blue is much more soluble in chloroform than the dye salt, and that the ratio of free base/salt of cresyl blue increases with the rise in the pH value. The dye X, on the other hand, appears pinkish red in chloroform. The amount of X absorbed by the chloroform is so much less than that of the free base of cresyl blue that it becomes visible only when the experimental condition is favorable (*i.e.*, at a high concentration and low pH value). For example, on shaking 0.07 per cent cresyl blue solution at pH 5.5 with the chloroform, the dye appears red in chloroform. On shaking with artificial sap, the red dye is not removed from the chloroform; in fact only a trace of the blue dye appears in the sap, which may very well be a trace of the cresyl blue extracted from the chloroform. On shaking 0.005 per cent cresyl blue solution at pH 9.2 or at pH 5.5, or 0.07 per cent cresyl blue solution at pH 9.2, or 0.025 per cent cresyl blue solution at pH 6.8 with chloroform, the dye absorbed appears orange in chloroform. But when this chloroform is shaken with the sap, until the orange dye is completely removed from the chloroform, the red dye becomes visible in the chloroform, and is not extractable by the sap from the chloroform. This red dye is absorbed by the chloroform to about the same extent from pH 9.2 or from pH 5.5 cresyl blue solution, but the amount absorbed increases with the rise in the concentration of aqueous cresyl blue solution. The amount of X absorbed is so small, however, that the absorption causes only a very slight decrease in the concentration of the total dye solution even when the chloroform is shaken with 0.07 per cent cresyl blue solution at pH 5.5. These experiments are being continued and their results will be reported in later publications.

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[12] Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 1928.

Spectrophotometric measurements of these solutions of cresyl blue show that they give absorption curves characteristic of cresyl blue.

The free base of cresyl blue was obtained as follows: 0.005 per cent cresyl blue solution at pH 9.2 was shaken with chloroform. The chloroform was then carefully freed from the aqueous solution and immediately placed in a pyrex glass vial and caused to evaporate rapidly by bubbling air through the solution. The freshly collected sap was then placed in this glass vial, and the concentration of the dye was determined colorimetrically by comparison with the standard dye solutions of known concentrations. There was a small amount of the dye residue which was not readily soluble in the sap, and which was therefore discarded. The pH value of the sap containing the dissolved dye was at once determined by the glass electrode.

To ascertain whether this dye was contaminated by other alkaline substances absorbed by the chloroform from the buffer solution which might alter the pH value of the sap the following experiments were made. The chloroform was shaken with the buffer solution at pH 9.2 containing no dye and was evaporated in the vial as before. The freshly extracted sap was placed in the vial and the pH value was immediately determined by the glass electrode. Since the pH value of the sap collected in this vial was the same as that of the sap collected in a control vial (which had not come in contact with the chloroform) it is apparent that the chloroform does not absorb any alkaline substance from the buffer solution in the absence of dye which would alter the pH value of the sap. Whatever alteration occurs in the pH value of the sap in presence of the dye must therefore be due to the dye alone.

It may be asked whether this dye is actually free base of cresyl blue. It cannot be a pseudo base, since it is very soluble in water and is readily reversible, forming a salt at lower pH values (appearing blue), while the pseudo base is not readily soluble in water, in which it appears colorless, and is irreversible. On shaking this dye solution (made up in buffer mixtures) with chloroform, the behavior is found to be very much like that of the original cresyl blue solution. The spectrophotometric determination shows that the absorption curve of the dye taken up by chloroform is identical with that of brilliant cresyl blue. It would therefore appear that the dye represents primarily the free base of brilliant cresyl blue. A detailed account of these results will be subsequently published.

Condition of Cells before Experiments.—As previously described,[13] single cells were cut from the central portion of the plant and kept about 20 hours in pans of tap water at about 20°C. in a well ventilated room in diffused light. The cells which have suffered severe injury at the time of cutting die over night and those which may be slightly injured may recover so as to be suitable for use. The effect of cutting and of the subsequent treatment of the cells as described in connection with electrical measurements[14] is not manifested in the same way in the determination of penetration of dyes into the vacuole, so that it is not necessary, for example, to keep each cell in a separate test-tube for a day or more before an experiment. The precaution, however, was always taken of placing only a few cells in a large pan of water so as to minimize any possible effect of the sap diffusing from the dead cells to the living cells.

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[13] Regarding the care of cells and detection of injury see Irwin, M., *J. Gen. Physiol.*, 1925-28, **8**, 147; 1926-27, **10**, 271, 927; 1927-28, **11**, 123; 1928-29, **12**, 147.

Experiments on Penetration.—Cells were placed in solutions with one end (only a very small portion, just enough to avoid contamination) exposed to the air in a moist chamber, at the temperature at which the glass electrode measurement was subsequently made. The cells were kept in the dye solution about 15 minutes. Leaving one end of the cell out of the solution for this period in a moist chamber was not injurious since cells kept in this way in tap water containing no dye even after 8 hours showed no signs of injury.

Extraction of the Sap.—The cells were removed from the solution, rinsed rapidly in distilled water and wiped gently first with a soft dampened cheese cloth and then with the dry cloth. Then each cell was cut open by a pair of scissors (the cut being made at the end which projected out of the solution) and the sap was allowed to flow out into a pyrex glass vial (small enough to prevent evaporation). By thus extracting the sap from the unstained portion of the cell, it was possible to avoid contamination of the sap by dye diffusing out of the cell wall at the time of extraction. Two successive extractions of sap were made from the vacuole of each living cell. The first extraction, called for convenience "sap," was made without pressing on the cell wall, while the second extraction called "sap mixture" was made by squeezing the cell wall vigorously so as to press the protoplasm into the vacuolar sap. The pH value of the sap was found to be about 0.6 pH lower than that of the sap mixture. It took about 15 minutes for one person to collect the sap and the corresponding sap mixture sufficient for analysis. For each reading by the glass electrode method it required about 0.4 cc. of sap collected from the vacuoles of 40 or more cells. The extraction was made at the same temperature as the temperature at which the electrode measurement was made.

Standardization of the Glass Electrode and Method of Measurement.—The apparatus used is identical with the one described by MacInnes and Dole[15] but the method of standardization of the glass electrode and the calculation of the pH value of the unknown solution used was different. The standard solution of the M/20 potassium hydrogen phthalate was made up to pH 3.97 and its pH value was determined by means of the hydrogen electrode. This solution was made up every

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[14] Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929–30, **13**, 467.

[15] For reference see footnote 10.

2 weeks to keep the pH value constant. Due to the very low temperature coefficient of this solution the alteration in the pH value with varying temperature is negligible in the range in which the experiments were carried out (between 20° and 23°C.).

Before each series of experiments the temperature was noted, and the glass electrode was standardized by determining the potential of the standard phthalate solution[16] at pH 3.97. Then the pH value of the standard M/15 phosphate buffer solution at pH 6.647 (the pH value of which was checked by the hydrogen electrode) was determined by means of the glass electrode to ascertain the behavior of the electrode; in no case did the reading vary more than ±0.03 pH. The potential of the unknown solution was then determined, and the pH value of the unknown solution was calculated for the given temperature according to the usual formula[17]

$$\mathrm{pH}_m = \mathrm{pH}_n + \frac{E_m - E_n}{\frac{RT}{F}}$$

where pH_m denotes the pH value of the unknown solution and pH_n that of the standard, while E_m represents the potential of the unknown solution and E_n that of the standard (the value of $\frac{RT}{F}$ at different temperatures is given by Clark[11]).

The temperature during the day did not usually vary more than ±0.5°C. but in case a variation greater than ±0.5°C. occurred, the electrode was re-standardized. From day to day the temperature varied between 20° and 23°C., but this variation did not bring about errors, since the glass electrode was always standardized and a temperature correction was made accordingly. After each series of experiments the electrode was again standardized with the phthalate and the phosphate buffer to see whether the electrode remained unaltered during the experiments. In many cases where the test solution is much less buffered than the standard phosphate solution, it may be desirable to check the glass electrode before and after a series of experiments with a standard solution which is buffered to about the same extent as the test solution. This was done in the case of the *Nitella* sap, by using M/150 phosphate buffer solution at pH 5.5, or the control sap, whenever there was a possibility of the effect of the sap in presence of the dye on the electrode in a series of experiments. In the case of all the experiments presented in the text the electrode remained unchanged through each series of experiments, in that there was no variation of more than ±0.05 pH value with these standard solutions.

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[16] For review and bibliography on the glass electrode methods see Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 1928.

[17] The equation is taken from the one described by Kerridge, P. T., *Biochem. J.*, 1925, 19, 611.

Three electrodes were employed for the experiments described in the text. In no case did the pH value of the standard phosphate buffer solutions determined by these electrodes vary more than ±0.03 pH.

Method of Washing the Glass Electrode when Successive Determinations Are to be Made.—When the glass electrode is not in use, it is kept in 0.1 N HCl. When two measurements with different samples are to be made in rapid succession washing with distilled water (without washing with the acid) is adequate if the test solution is such that it does not measurably affect the electrode or the reading, or if the solution is sufficiently buffered, but if the test solution does affect the electrode and if it is not sufficiently buffered, the alteration will result in a wrong pH value. An example of such cases may be cited as follows. The pH value of a strongly buffered solution, such as standard phosphate buffer at pH 6.647, in presence or absence of basic dye, or that of the distilled water containing no dye, may be measured repeatedly without showing any effect of the test solution on the electrode whether the electrode is washed after each measurement with 0.1 N HCl and then with distilled water or only with distilled water. But if the distilled water contains, for example, 0.07 per cent cresyl blue in form of salt or free base, an effect on the electrode is manifested by an increase in the pH value after each successive measurement if the electrode is washed only with distilled water each time. But such an effect is not manifested if the electrode is first washed with acid each time before applying distilled water.

The freshly extracted sap is more buffered than the distilled water but not sufficiently so to enable us to omit the washing with acid each time, even though the electrode is always washed with the test sap before each measurement. If the electrode is not washed with the acid there is a small but progressive increase in the observed pH value of sap containing cresyl blue (in form of salt or in form of free base) with each successive drop of the same sample of sap taken for each one of the five measurements, where the maximum increase is a little over 0.1 pH. The observed pH value of the same sap remains constant throughout such a series of experiments when the electrode is washed with 0.1 N HCl and then with distilled water followed by final washing with test solution after each measurement. There is no danger of the acid diffusing out of the electrode which has been washed with acid into the sap, since the pH value of the sap containing no dye is found to be the same whether the electrode is first washed with the acid or not.

To avoid the errors arising from this source, the following procedure was adopted in the experiments described in the text. Before using the electrode it is removed from 0.1 N HCl, wiped with filter paper, washed in distilled water, and wiped again. Then the electrode is washed quickly in the solution, the potential of which is to be determined. After measurement, the electrode is washed in distilled water, wiped, and placed in 0.1 N HCl until the effect of the test solution on the electrode is completely removed. When the electrode is not in use, it is always kept in the 0.1 N HCl. The temperature of the solutions used to wash the electrode is kept within ±0.5°C. of the temperature of the test solution.

Time Curve for Glass Electrode Measurement.—It is always necessary to continue measurement for about 5 or 10 minutes to see if there is any change in the potential with time. Such a change may occur from the effect of the solution on the electrode or from the change in the solution proper. The results show that there is no alteration in the pH value of the buffer solution at pH 6.65 within 5 minutes, nor in the pH value of the conrrol sap. There is a tendency for the pH value of the sap containing the dye to rise with time, but the maximum rise in 5 minutes is only 0.05 pH, which is within an experimentl error. The two measurements made within 2 minutes agree within ±0.03 pH; the measurements given in the text represent the average of two readings made, (*a*) one-half minute after the electrode has first come in contact with the test solution, and (*b*) one minute and a half later.

The pH Value of the Control Sap or the Sap Mixture. If the pH value of the control sap changed rapidly after extraction, it would interfere with such experiments as are described in the text, but fortunately there was no alteration in the pH value of the sap nor of the sap mixture even on standing in a pyrex glass vial (stoppered) 2 hours after extraction. Even on keeping the sap in the refrigerator for 20 hours the pH value of the sap was found to increase only 0.2 pH.

Furthermore there was practically no alteration in the pH value of the sap nor of the sap mixture during the 15 minutes required for extraction. This test was made colorimetrically,[18] since by this method only a small capillary tube of sap was sufficient so that it was possible to complete the first determination only 3 minutes after the extraction had begun. The indicator method was found to be adequate since the pH value of the sap determined by this method agreed closely enough (within ±0.1 pH) with the pH value determined by the glass electrode.

From day to day there was some variation in the pH value of the control sap, the maximum variation being about ±0.2 pH. Owing to this an alteration in the pH value of the sap less than 0.2 pH should not be taken seriously. Though the pH value of the sap did not alter on standing for several hours, the measurement with the glass electrode was always made immediately after the sap was extracted.

Each reading given in the text is an average of 10 to 15 experiments employing altogether 400 cells or more.

III

Nitella flexilis (*A*)

The Sap.—The average sap of the control cells (taken from tap water) is at about pH 5.36 (Table 1).

When the cresyl blue salt at a concentration in the neighborhood of 0.07 per cent is dissolved in the sap *in vitro* the pH value of the sap is found to decrease by about 0.07 pH, which is too small an alteration to be considered seriously (Table I).

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[18] For method see Irwin, M., *J. Gen. Physiol.*, 1925–26, **9**, 240, Section III.

When about 0.07 per cent free base [19] of cresyl blue is dissolved in the sap *in vitro*, the pH value is found to increase by about 0.55 (Table I).

TABLE 1

pH Value of the Sap of Living Cells of Nitella flexilis (A)

In presence of cresyl blue. Average temperature 21°C. Electrode I was used. Unless otherwise stated, penetration experiments were carried out with one end of the cell outside the solution in moist chamber, to avoid contamination of the sap. The duration of the experiments was about 15 minutes.

	Conc. of dye in sap in per cent	pH of sap	Increase in pH of sap over the control sap	Conc. of dye in sap containing protoplasm in per cent	pH of sap containing protoplasm	Increase in pH of sap containing protoplasm over the control sap containing protoplasm
Control cells in tap water..........	0	5.36		0	5.97	
Dye salt added *in vitro*............	0.069	5.29				
Free base of dye added *in vitro*......	0.071	5.91	0.55	0.070	6.48	0.51
Penetration from 0.002 per cent dye at pH 9.2......................	0.073	5.86	0.50	0.065	6.45	0.48
Penetration from 0.025 per cent dye at pH 6.8......................	0.071	5.87	0.51	0.074	6.37	0.40
Penetration from 0.004 per cent dye at pH 9.2......................	0.18	6.66	1.30			
Free base added *in vitro*...........	0.16	6.50	1.24			
Penetration from 0.002 per cent dye at pH 9.2, when cells are completely immersed..............	0.070	5.88	0.52			
After 8 hours in tap water containing no dye....................	0	5.40		0	6.00	
After 1 hour in buffer solution at pH 9.2, containing no dye..........	0	5.37		0	5.94	

These results prove that the two forms of the dye are distinguishable by determining the pH value with the glass electrode, the dye salt having very little effect on the sap, the free base of the dye distinctly raising the pH value of the sap. If therefore the same amount of dye penetrating the vacuole raises the pH value to about the same extent as the free base of the dye, we may conclude that the dye penetrates as free base and not as salt. The following results show this to be the case.

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[19] For method see Section II in the text.

When cells are placed[19] for about 15 minutes in 0.002 per cent dye solution at pH 9.2 until about 0.07 per cent dye has accumulated in the vacuole, the pH value of the sap is found to increase about 0.5 over that of the control sap (Table I). This increase[20] agrees closely with the increase obtained by dissolving 0.07 per cent free base of the dye in the sap (Table I), thus indicating that the dye penetrates chiefly as free base and not as salt.

Previous experiments[21] have shown that at pH 9.2 cresyl blue exists predominantly as free base so that it would not be surprising if the dye penetrated as free base from this solution. But at a lower pH value, where the dye salt predominates, does the dye enter as salt or as free base? To test this point the dye was allowed to penetrate for about 15 minutes from 0.025 per cent cresyl blue solution at pH 6.8 (in which there was a preponderance of the dye salt).[21] As shown in Table I, 0.07 per cent dye penetrating from this solution has brought about approximately the same increase (about 0.51 pH) in the pH value as 0.07 per cent dye penetrating from the solution at pH 9.2. These results prove that irrespective of the ratio of the free base to salt of the dye in the external solution, the dye penetrates as free base and not as salt.

The dye was furthermore allowed to penetrate to a still higher concentration (0.18 per cent) from 0.004 per cent cresyl blue solution at pH 9.2 in about 15 minutes, to see if further increase in the pH value of the sap would occur with a higher concentration of the dye in the sap. An increase[20] of about 1.30 pH over the control was found to take place, which agreed closely with the rise in the pH value by dissolving 0.16 per cent free base of cresyl blue in the sap *in vitro* (Table I).

The pH value of the sap in the presence of a lower concentration of cresyl blue (below 0.07 per cent) was determined but owing to the variation in the pH value of the control sap (to the extent of 0.2 pH) it is not desirable to draw conclusions from the lower concentration of the dye in the sap which brought about this increase in the pH value of the sap.

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[20] These results prove that the alteration in the rate of entrance and exit of the dye formerly reported to occur in the cell after penetration of dye are not due to the production of acid in the sap to the extent of lowering the pH value of the sap below the normal. Irwin, M., *J. Gen. Physiol.*, 1925–28, **8**, 147; *Proc. Soc. Exp. Biol. and Med.*, 1926–27, **24**, 247.

[21] For reference see footnote 3.

Owing to the fact that these experiments were carried out when a portion of the cell was exposed to the air in a moist chamber (to avoid contamination of the sap from the stained cell wall at the time of extraction) the question may be raised whether the increase in the pH value of the sap may not be caused by the penetration of the alkaline buffer as a result of injury to the cells under these experimental conditions. This "partial immersion" method, however, is proved to cause no injury in the duration of these experiments, as shown by the following experiments.

When the experiments were repeated by completely immersing the cells in 0.002 per cent dye at pH 9.2 for a little less than 15 minutes until 0.07 per cent dye had collected in the sap, the pH value of the sap was found to increase about 0.52 pH, which agrees closely with the rise brought about when cells were only partly immersed in the solution as previously stated (Table I). In this dye solution at pH 9.2 so little staining of the cell wall occurs (with this length of exposure) that no question of errors arising from contamination of the cell sap from the stained cell wall enters in the case of the cells completely immersed in this solution. If the partial immersion method causes injury, thereby hastening the penetration of the alkaline borate buffer, the rise in the pH value of the sap may be expected to be greater when this method is used than the rise when the cells are completely immersed. Since the rise in the pH value is about the same in both cases, we may conclude that the alteration in the pH value of the sap is not due to injury caused by the partial immersion method.

Furthermore the pH value of the cells which were partially immersed in this way in tap water for 8 hours or in the buffer solution at pH 9.2 (containing no dye) for 1 hour was the same as that of the control sap, thus showing that the partial immersion method does not injure the cells (for the duration of exposure described in the text) enough to affect the pH value of the sap (Table I).

Sap Containing Protoplasm.—The most striking fact is that after the sap has been allowed to flow out without pressure, if the cell wall is squeezed so as to press the protoplasm into the sap, the pH value of this mixture is found to be about 0.6 pH higher than the sap proper (Table I). This may be chiefly due to the presence of the protoplasm, the pH value of which may be higher than that of the sap. Another possible explanation is that the fluid[22] which may exist in the space between the protoplasmic layer and the cell wall may also be squeezed into the sap and may help to raise the pH value of the sap.

In presence of 0.07 per cent cresyl blue free base the pH value of this mixture is increased about 0.51 over that of the control mixture (Table I). The cresyl blue penetrating from the dye solution at pH 9.2 or at pH 6.8 also raises the pH value of sap containing protoplasm by about 0.4 to 0.5 pH (Table I), thus showing that the dye penetrates chiefly as free base into this mixture. Undoubtedly not all the protoplasm can be squeezed into the sap and the protoplasmic layer is very thin so that in the mixture the sap preponderates over the protoplasm. For this reason it would be difficult to conclude from these results whether the dye penetra'es chiefly as free base into the protoplasm as it does into the vacuole.

IV

Nitella flexilis (*B*)

To determine if the penetration of cresyl blue as free base will occur with another sample of *Nitella* obtained in a different locality, the experiments were repeated with cells collected elsewhere which are distinguished for convenience from *Nitella* (*A*) by the term *Nitella* (*B*).

The Sap.—The average pH value of the sap of *Nitella* (*B*) is pH 5.6 (Table II), which is about 0.2 pH higher than the pH value of the sap of *Nitella* (*A*).

On dissolving the 0.086 per cent free base[19] of cresyl blue in this sap *in vitro* the pH value of the sap is found to increase about 0.37 pH (Table II). This increase is about 0.2 pH less than is the case with *Nitella* (*A*). This may mean that the sap of *Nitella* (*B*) is more buffered than the sap of *Nitella* (*A*) (Tables I and II). On adding the dye salt to the sap there is practi [illegible] teration in the pH value of the sap. When about 0.075 per cent dye penetrates the vacuole from the cresyl blue solution at pH 9.2 or 6.8, there is an increase in the pH value of 0.4 or 0.45 pH over that of the control sap (Table II), which agrees closely with the rise in the pH value when the free base of the dye is dissolved in the sap *in vitro*. In this case the increase in the pH value is a little less (about 0.1 to 0.2 pH) than that of the *Nitella* (*A*).

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[22] Irwin, M., *J. Gen. Physiol.*, 1926–27, **10**, 75.

TABLE II

pH Values of the Sap of Living Cells of Nitella flexilis (*B*)

In presence of cresyl blue. Average temperature at 22°C. Electrode I was used unless otherwise stated. The penetration experiments lasted about 15 minutes (with precautions to avoid contamination of the sap).

	Conc. of dye in sap in per cent	pH of sap	Increase in pH of sap over the control sap	Conc. of dye containing protoplasm in per cent	pH of sap containing protoplasm	Increase in pH of sap containing protoplasm over the control sap containing protoplasm
Control	0	5.60		0	6.11	
Free base of dye added *in vitro*	0.086	5.97	0.37	0.077	6.43	0.32
Dye salt added *in vitro*	0.080	5.55		0.085	6.07	
Penetration from 0.002 per cent dye at pH 9.2	0.076	6.00	0.40	0.065	6.35	0.24
Penetration from 0.025 per cent dye at pH 6.8	0.077	6.05	0.45	0.069	6.36	0.25
Penetration from 0.01 per cent dye in tap water	0.076	5.96	0.38	0.072	6.37	0.26
Cells placed for ½ hour in buffer solution at pH 9.2 after cells had been in 0.002 per cent dye at pH 9.2 for about 15 minutes	0.075	5.95	0.35	0.070	6.31	0.20
Penetration from 0.002 per cent dye at pH 9.2 Electrode II	0.079	5.98	0.38	0.070	6.39	0.28
Penetration from 0.002 per cent dye at pH 9.2 Electrode III	0.074	5.95	0.35	0.068	6.36	0.25

Thus the results on *Nitella* (*B*) confirm those on *Nitella* (*A*), showing that in both cases the dye penetrates the vacuole chiefly as free base.

The Sap Containing Protoplasm.—The pH value of the mixture of the sap and the protoplasm extracted as before behaved very much like the mixture of *Nitella* (*A*) in that the pH value of the mixture is raised when the free base of the dye is dissolved in the mixture *in vitro* (Table II). On examining the pH values of the mixture after penetration of the dye they are found to be 0.1 to 0.2 pH lower than the corresponding pure sap containing approximately the same amount of dye (Table II). Such results may signify that some dye salt[23] penetrates into the protoplasm or that the pH value of the protoplasm is decreased when the dye penetrates as a result of production of acid by the protoplasm.

Though the results definitely show the penetration of cresyl blue into the sap to be chiefly as free base there is still one more uncertainty to be removed and this is in relation to the possibility of the rise in the pH value of the sap taking place as a result of penetration of an alkaline substance other than the dye owing to the injury caused by the penetration of the dye. Such a possibility is imminent owing to previous[20] results showing changes in the rate of penetration from the normal as a result of penetration of over 0.006 per cent dye. Since the concentration of cresyl blue in the vacuole in the present case is high (about 0.07 per cent), it is necessary to prove that the increase in the pH value of the sap is not due to the penetration of an alkaline substance other than the dye into those cells the condition of which is altered from the normal by penetration of the dye.

Since in all the experiments the length of exposure and the concentration of the dye which has penetrated into the sap are approximately equal, if the increase in the pH value of the sap were due to the penetration of an alkaline substance other than the dye, there should be a greater increase in the pH value of the sap when cells are exposed to the dye solution at pH 9.2 than at pH 6.8 or in tap water at pH 7.7. But this increase is found to be about constant irrespective of the external pH values and the external alkaline buffer concentrations, provided the concentration of dye in the sap is approximately the same (Table II). These results therefore point to the fact that whatever secondary changes that might occur in the cell they do not admit an alkaline substance other than the dye into the vacuoles of these cells.

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[23] Experiments on penetration of dye from methylene blue, described in the subsequent paper, show that such a lowering is not due to the penetration of the dye salt, so that in all probability the same is true with the cresyl blue. Irwin, M., *J. Gen. Physiol.*, 1930–31, **14**, 19.

Another proof is as follows. If the increase of 0.4 pH in the sap over the control sap is not due to the penetration of alkaline buffer as a result of secondary changes, but is due to the penetration of about 0.08 per cent cresyl blue free base from the dye solution at pH 9.2, then by transferring such cells from the dye solution into the buffer solution at the same pH value containing no dye there should be no progressive increase in the pH value of the sap. Such is found to be the case experimentally, as shown in Table II, when even after ½ hour exposure of these cells to the buffer solution at pH 9.2 there is no further increase in the pH value of the sap. But a further increase is found to occur if more dye penetrates the vacuole.

These experiments were made with one electrode (I) only: afterward check experiments were made with several electrodes, which gave about the same results as Electrode I. The results from two other electrodes are recorded in Table II.

The spectrophotometric measurements show that the dye which has penetrated from the cresyl blue solution into the sap or into the sap mixture is identical with the external dye solution as well as the cresyl blue free base dissolved in the sap *in vitro* in that the absorption curves are characteristic of cresyl blue.

V

CONCLUSION

From these results we may conclude that the cresyl blue penetrates chiefly as free base and upon coming in contact with the sap at the vacuolar surface it is at once partly converted into the dye salt. At equilibrium the concentration of free base in the sap is proportional to that in the external solution. Thus the rate of penetration of the dye in form of free base and the concentration of the total dye (free base and salt) in the sap at equilibrium are dependent on the dissociation constant of the dye. For this reason both the rate of penetration and the concentration of the dye in the sap at equilibrium are increased with a rise in the external pH value (which increases the concentration of free base of the dye) or with a decrease in the pH value of the sap (which increases the conversion of the free base to the dye salt).

It is possible that some of the dye may be converted to something different from the dye salt after the dye has penetrated the vacuole. Previous experiments[20] have shown that as the concentration of the dye in the sap through penetration reached a certain point the rate of penetration progressively increased while that of the exit decreased from that of the normal. Such an alteration in the rate is not due to the excess production of acid in the sap as shown by the preceding results. It may be caused by some kind of change in the dye as the dye penetrates the sap. Such a dye may be either another tautomer of cresyl blue, or a compound formed between the cresyl blue and the sap, or another dye. To affect the rate, as already stated, the reaction producing this dye must be less reversible than that producing cresyl blue salt or this dye must have a lower rate of exit from the vacuole into the external solution than cresyl blue salt.

These results cannot be due to exchange of dye cations for hydrogen ions, as this would involve a lower rate of penetration since the surfaces are non-aqueous and the concentration of hydrogen ions in the sap is too low. This will be discussed in subsequent papers.

SUMMARY

Glass electrode measurements of the pH value of the sap of *Nitella* show that cresyl blue in form of free base penetrates the vacuoles and raises pH value of the sap to about the same degree as the free base of the dye added to the sap *in vitro*, while the dye salt dissolved in the sap does not alter its pH value. It is proved conclusively that the increase in the pH value of the sap is due only to the presence of the dye and not to some other alkaline substance.

Spectrophotometric measurements show that the dye which penetrates the vacuole is chiefly cresyl blue.

When the protoplasm is squeezed into the sap, the pH value of the sap is higher than that of the pure sap. Such a mixture behaves very much like the sap in respect to the dye.

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STUDIES ON PENETRATION OF DYES WITH GLASS ELECTRODE

V. WHY DOES AZURE B PENETRATE MORE READILY THAN METHYLENE BLUE OR CRYSTAL VIOLET?

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I

The results[1] from measurements made with the glass electrode and spectrophotometer show that brilliant cresyl blue penetrates as free base into the vacuoles of living cells of *Nitella*. The present paper describes similar experiments with azure B, methylene blue, and crystal violet.

Since the methods for the condition of cells, extraction of the sap, penetration experiments, extraction of free base of dyes, making up of dye solutions, and measurements by means of the glass electrode have been published[1] in detail, they are omitted here. The dyes were obtained as salts. The azure B (in pure form) was made by W. C. Holmes of the Color Laboratory, Washington, D. C., methylene blue (medicinal) by Merck and Co., crystal violet by Grübler (obtained before 1914). These dyes affected the electrode slightly more than cresyl blue (causing the pH value of the sap containing the dye to increase between 0.1 and 0.2 pH value after the fifth measurement (when the electrode was not washed with acid), but these effects were entirely eliminated by washing the electrode with acid as already described.[1] Just as with cresyl blue, the readings did not vary within 5 minutes after the electrode was brought in contact with any one of these dyes.

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[1] Irwin, M., *J. Gen. Physiol.*, 1930–31, **14**, 1.

II

Azure B or Trimethyl Thionin

A. Nitella (A)

The Sap.[1]—When about 0.07 per cent azure B salt is dissolved in the sap *in vitro* the pH value remains practically the same as that of the control (Table I), but when the same concentration of the dye in the form of free[1] base is dissolved in the sap *in vitro* the pH value is found to be about 1 pH above that of the control sap (Table I). Owing to this difference it is possible to find out which form enters the vacuoles of living cells of *Nitella* by determining the pH value of the sap after penetration.

When cells are placed in 0.01 per cent azure B solution at pH 9.2 for about 15 minutes until 0.07 per cent dye has penetrated the vacuole, the pH value of the sap is found to increase about 1 pH above that of the control sap (Table I).

Since azure B which has penetrated the vacuole increases the pH value of the sap to about the same extent as the same concentration of the dye in form of free base added to the sap *in vitro*, while the dye salt dissolved in the sap *in vitro* does not alter the pH value of the sap, it would seem that the azure B penetrates chiefly as free base and not as salt.

But the experimental results supporting this conclusion are not so numerous as with cresyl blue.[1] There is a greater possibility of injury to cells exposed to azure B than to cresyl blue. Since the rate of penetration at pH 9.2 is much slower with azure B than with cresyl blue, it was necessary to use a higher concentration[2] of azure B in order to maintain a comparable length of exposure to both these solutions. Cells were dead in this azure B solution after 3 hours' exposure while these cells in the cresyl blue solution were not dead until after 6 hours' exposure. Since injury hastens the entrance into the vacuole of substances whose penetration under normal conditions is negligible it is all the more necessary to ascertain whether the cells were injured to such an extent that alkaline substances other than the azure B penetrated. Unfortunately it was not possible to determine this point so

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[2] A lower concentration of azure B at pH 9.2 (causing penetration of 0.03 per cent dye in 15 minutes) was employed in which a rise in the pH value of the sap was found to be at about 0.5 pH over that of the control, and in which cells lived somewhat longer than in the solution described in the text. But even in this case, the results were not so conclusive as with cresyl blue.

TABLE I

pH Values of the Sap of Living Cells of Nitella flexilis. (A and B)

In presence of azure B and methylene blue. Average temperature 22.5°C. Duration of experiments was about 15 minutes with azure B and about 80 minutes with methylene blue (precaution was taken to avoid contamination of the sap). Electrode I was used unless otherwise stated.

	Conc. of dye in sap in per cent	pH of sap	Increase in pH of sap over the control sap	Conc of dye in sap containing protoplasm in per cent	pH of sap containing protoplasm	Increase in pH of sap containing protoplasm over the control sap containing protoplasm
			Nitella A, azure B			
Control	0	5.25		0	6.09	
Dye salt added *in vitro*	0.07	5.27	0.02	0.070	6.14	0.06
Free base of dye added *in vitro*	0.73	6.37	1.10	0.080	7.20	1.06
Penetration from 0.01 per cent dye at pH 9.2	0.075	6.39	1.14	0.070	6.73	0.64
			Nitella B, azure B			
Control	0	5.52			6.00	
Penetration from 0.01 per cent dye at pH 9.2	0.08	6.52	1.0	0.080	6.50	0.5
Penetration from 0.01 per cent dye at pH 9.2. Electrode II	0.08	6.45	0.93	0.075	6.43	0.43
			Nitella A, methylene blue			
Control	0	5.30		0	6.10	
Dye salt added *in vitro*	0.07	5.28		0.075	6.17	0.07
*Free base added *in vitro*	0.067	6.31	1.01	0.065	6.99	0.89
Penetration from 0.04 per cent dye at pH 9.2	0 07	6.43	1.13	0.073	6.78	0.61

* This free base is azure B extracted from methylene blue solution at pH 9.2.

satisfactorily as with cresyl blue. Owing to the heavy staining of the cell wall when cells were placed in the azure B solution (at all pH values) the dye at once began to diffuse into the vacuoles from the cell wall when cells were transferred from the dye solution at pH 9.2, for example, into the buffer solution at the same pH value containing no dye so that it was not possible to prove whether or not the pH value of the sap would continue to increase without further penetration of the dye. Furthermore it was not possible to determine the penetration of azure B from tap water in the given period without employing a very high concentration of dye (too high for experiments without danger of causing injury to cells).

Thus the proof of the penetration of the dye in form of free base was less conclusive than with cresyl blue, but it seems probable that the azure B also penetrates as free base.

Sap Mixture[1] *(Sap Containing Protoplasm)*.—Addition of 0.07 per cent dye salt or the free base to the mixture of sap and protoplasm produces much the same effect as on sap in that the dye salt brings about practically no alteration in the pH value of the mixture while the free base of the dye increases the pH value about 1 pH over that of the control mixture (Table I). But when the azure B penetrates until 0.07 per cent dye has collected in the vacuole, the increase in the pH value of this mixture is found to be only about 0.6 pH instead of 1 pH as was the case with the sap. Judging from the results obtained with penetration of dye from methylene blue (described in following section) we may conclude that this difference may be due to the production of acid by the protoplasm as a result of penetration of dye, and not to the penetration of azure B salt into the protoplasm. It is not due to the greater buffer action of the protoplasm since the pH value is increased to about the same extent when the azure B free base is added *in vitro* to the sap or to the mixture of the sap and the protoplasm. As the protoplasm consists of only a very thin layer it is not possible to obtain it free from the sap so as to study its behavior.

B. Nitella (B)

Approximately the same results were obtained when the experiments were repeated with *Nitella*[1] *(B)* (Table I).

On employing Electrode II, the same results were obtained (Table I).

Spectrophotometric measurements show that the dye which has penetrated the vacuole is like the free base dissolved in the sap *in vitro*, and the external dye solution employed in giving absorption curves characteristic of azure B with primary absorption maxima at about 650 mμ.

III

Methylene Blue or Tetramethyl Thionin

Nitella (*A*)

The Sap.[1]—The pH value of the sap after 0.07 per cent dye had penetrated from 0.04 per cent methylene blue solution at pH 9.2 in 80 minutes was found to be about 1 pH higher than the control (Table I), which agreed closely with the rise in the pH value brought about by dissolving the 0.07 per cent free base of azure B in the sap (Table I). The methylene blue chloride when dissolved in the sap brought about practically no change in the pH value of the sap (Table I).

There was a striking similarity in the behavior of the dye absorbed by the vacuole and by the chloroform. When chloroform was shaken up with the methylene blue solution at pH 9.2, the dye appeared red in the chloroform. After evaporation of the chloroform, the dye residue was dissolved in sufficient sap to make the concentration 0.07 per cent. The pH value of this sap was found to be again about 1 pH higher than that of the control sap (Table I).

On determining by means of the spectrophotometric measurements the nature of the dye which has penetrated the vacuole from this methylene blue solution until 0.07 per cent has collected in the sap, the absorption curves showed that the dye consisted more of azure B than methylene blue with primary absorption maxima at 653 to 655 mμ. The dye absorbed by the chloroform was also found to be chiefly azure B with a primary absorption maximum at 650 mμ.

These results therefore confirm those obtained previously by spectrophotometric analysis[3,4] and support the conclusion previously made that the azure B free base present as impurity in methylene blue solution penetrates the vacuole so much more rapidly than methylene blue salt that the dye which collects in the vacuole consists chiefly of azure B and not methylene blue.

[3] Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926–27, **24,** 425; 1927–28, **25,** 563; *J. Gen. Physiol.*, 1928–29, **12,** 147, 407.

[4] Experiments on spectrophotometric measurements were repeated by Brooks, who found methylene blue to penetrate instead of azure B. Brooks, M. M., *Proc. Soc. Exp. Biol. and Med.*, 1928–29, **26,** 290. Protoplasma, 1929, **7,** 46.

As soon as cells are injured, however, the methylene blue penetrates more rapidly so as to shift the absorption curve of the dye in the sap from that of a mixture containing more azure B than methylene blue to that of a mixture containing more methylene blue than azure B. Since in the present instance where there is 0.07 per cent penetration of dye from methylene blue solution at pH 9.2 it is the azure B free base that still penetrates predominantly, the cells thus exposed cannot be injured to the extent of permitting the dye salts to penetrate freely although the cells are dead in the methylene blue solution within three hours' exposure.

Owing to this toxicity of methylene blue solution and to the necessity of longer exposure of these cells to the solution, the results are not so convincing as those obtained on cresyl blue, but the evidence is in favor of the conclusion that predominantly azure B in form of free base penetrates the vacuoles unless cells are injured.

"Sap Mixture"[1] *(Sap Containing Protoplasm)*.—The same results as in the case of the sap were obtained when the methylene blue or the dye extracted by the chloroform from the methylene blue solution was dissolved in the sap mixture *in vitro* (Table I). But on penetration of the dye from the methylene blue solution at pH 9.2 into the vacuoles the pH value of the sap mixture was raised only 0.6 pH over that of the control sap mixture instead of 1 pH increase, as was the case of the sap. This difference is not due to the greater penetration of methylene blue into the protoplasm than into the sap, because the dye in the sap mixture gives the same absorption curve as the dye in the sap, with a primary absorption maximum at 653 to 655 mμ (characteristic of a mixture of azure B and methylene blue with preponderance of the former). In all probability it is due to the production of acid by the protoplasm as a result of the dye penetration.

IV

Crystal Violet

Nitella (A)

Crystal violet is more toxic than other dyes employed here and the rate of penetration at any pH value into the cells of *Nitella* is not sufficiently rapid to cause an adequate penetration of the dye before there is a danger of injury to cells. In 0.001 per cent dye solution, for example, the cells are dead after 1 hour, while the penetration after 15 minutes is too small to show whether the dye increases the pH value. The rate of penetration is approximately the same at pH 9.2 as at pH 5.5. As the dye penetrates, a violet precipitate appears in the sap with very little of the dissolved dye. The penetration of the dye is in all probability mostly due to injury. Crystal violet is a basic dye and it is soluble in chloroform so that according to Overton's theory it should penetrate the cells rather rapidly. Since the penetration is found to be slow, it is of interest to find out more about the behavior of this dye toward the sap of *Nitella in vitro* as a basis for an explanation of why it does not penetrate the vacuole readily.

When 0.07 per cent crystal violet chloride was dissolved in the freshly extracted sap, the pH value was not higher than that of the control sap (about pH 5.3).

The dye was absorbed by chloroform from aqueous solution at pH 5.5 and pH 9.2 in about the same amount. The stained chloroform was freed from the aqueous solution, allowed to evaporate, and the colored residue was dissolved in the sap. This did not raise the pH value of the sap above that of the control sap (whether the dye thus dissolved was obtained by shaking the chloroform with crystal violet solution at pH 5.5 or pH 9.2).

The behavior of crystal violet in sap is therefore different from that of the free base of cresyl blue or of azure B in that with crystal violet there is no difference between the dye salt and the dye absorbed by the chloroform from the solution at pH 9.2, while with cresyl blue or azure B the dye salt does not alter the pH value of the sap while the dye absorbed by the chloroform from the dye solution at pH 9.2 increases it.

Spectrophotometric determination shows that the absorption curves characteristic of crystal violet are obtained with the dye in buffer solutions at pH 9.2 or at pH 5.5, or with the dye which was absorbed by the chloroform from these solutions.

V

Theoretical Considerations

Do these facts help us to understand why azure B enters the vacuoles of living cells of *Nitella* rapidly while methylene blue and crystal violet penetrate very slowly?

The results show that in aqueous solution azure B exists chiefly in two forms, the dye salt and free base, while methylene blue and crystal violet exist chiefly in one form. They also show that azure B penetrates chiefly as free base and not as salt. Since the free base of azure B predominates over the salt at higher pH values, the rate of penetration of the dye increases as the external pH value rises. The increase of free base over salt depends on the "apparent dissociation constant" of the dye.

As soon as the dye in form of free base passes through the protoplasm and comes in contact with the sap in the vacuole, the greater portion of it is converted into the dye salt. The extent of this conversion is dependent on the basicity of the dye, and on the constituents of the sap (such as the hydrogen ion, organic salts, and protein). With *Nitella* sap, the chief factor controlling the conversion is the pH value of the sap. Owing to the low pH value of the sap and to the apparent dissociation constant of azure B, the free base of the dye is converted to the salt to such an extent that only a very small proportion of the dye exists in the sap as free base. At equilibrium the concentration of free base of the azure B in the sap is proportional to that of the free base of the dye in the external solution, so that the higher the concentration of the free base outside, the higher is the concentration of the total dye (free base and salt) in the sap. This explains why at equilibrium the concentration of the total dye in the sap is found to be higher when the pH value of the external solution is raised or the concentration of the dye is increased.

Why is it that the free base of the dye penetrates more rapidly than the dye salt? In these cases the living cells of *Nitella* behave as if the rate of penetration of the dye into the vacuoles is controlled by three phases only, that is, the non-aqueous layer lying between the external aqueous phase and the internal aqueous sap in the vacuole. The rate of penetration is partly controlled by the partition coefficients of the dye at these two phase boundaries. Azure B free base, for example, diffuses into the non-aqueous layer readily because the partition coefficient K_e of this form of dye between the non-aqueous layer and the external solution is high. But no matter how high the K_e is, the dye cannot diffuse into the vacuole unless the partition coefficient K_i of the free base between the non-aqueous layer and the sap is low or the free base is largely converted by the sap into another form, the K_i of which is low. With azure B, the free base is predominantly converted to the salt, the K_i of which is low, so as to promote a rapid penetration and accumulation of the dye in the sap. The rate of penetration of dye will be greatly reduced by increasing the pH value of the sap and so causing less transformation of free base to the salt.

The exit of the dye may also be accounted for on the same theoretical basis which explains why the rate of exit is hastened with a decrease in the external pH value.

Methylene blue does not penetrate the vacuole readily because it exists as salt even at a high pH value such as pH 9.2, and the partition coefficient K_e of the dye salt is low.

The reason crystal violet does not penetrate the vacuoles readily, though the partition coefficient K_e is high, is because the partition coefficient K_i of the dye is also high. The results indicate that crystal violet exists only in one form in the range of pH values available for living cells of *Nitella* (between pH 5 and pH 9.2), so that there is no such transformation of free base into the salt as shown by azure B. It is uncertain whether the crystal violet exists as salt or as free base. From the structure it appears to be a strongly basic dye, but owing to its solubility in lipoid it may be a very weak base. In case it is a weak base, it is too weak to increase the pH value even of the distilled water.

Owing to the penetration being dependent (at least in part) on more than one partition coefficient, the theory was called the "multiple partition coefficient theory."[5] It is uncertain yet whether this non-aqueous layer is lipoid in nature but its behavior is very much like that of lipoid. This theory is not identical with Overton's even if the non-aqueous layer proves to be a lipoid because Overton considered only one partition coefficient, K_e, between the non-aqueous layer and the external solution. His theory is tenable in so far as those dyes which are not soluble in lipoid do not readily penetrate the vacuoles of these living cells, but it fails to explain why those dyes which are soluble in a lipoid do not readily penetrate. In the case of crystal violet, for example, it becomes explainable only when the second partition coefficient, K_i, is considered. Furthermore, the accumulation of the dye in the vacuole cannot be explained on the basis of his theory.

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[5] For the theory of multiple partition coefficients see Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1927–28, **25**, 127; *J. Gen. Physiol.*, 1927–28, **11**, 111; 1928–29, **12**, 147, 407.

Such instances show the simplest type of penetration. In more complicated cases we must consider other partition coefficients. In view of the fact that a living cell of *Nitella* consists of a heterogeneous system composed of an outer non-aqueous layer (in contact with the external solution), the middle aqueous layer of the protoplasm, the inner, non-aqueous layer (in contact with the sap in the vacuole), and the aqueous sap, the rate of penetration may be controlled by two or more of the four partition coefficients. The discussion of these cases will be deferred to a later publication.

SUMMARY

Glass electrode measurements of the pH value of the sap of cells of *Nitella* show that azure B in the form of free base penetrates the vacuoles and raises the pH value of the sap to about the same degree as the free base of the dye added to the sap *in vitro*, but the dye salt dissolved in the sap does not alter the pH value of the sap. It is concluded that the dye penetrates the vacuoles chiefly in the form of free base and not as salt.

The dye from methylene blue solution containing azure B free base as impurity penetrates and accumulates in the vacuole. This dye must be azure B in the form of free base, since it raises the pH value of the sap to about the same extent as the free base of azure B dissolved in the sap *in vitro*. The dye absorbed by the chloroform from methylene blue solution behaves like the dye penetrating the vacuole. These results confirm those of spectrophotometric analysis previously published.

Crystal violet exists only in one form between pH 5 and pH 9.2, and does not alter the pH value of the sap at the concentrations used. It does not penetrate readily unless cells are injured.

A theory of "multiple partition coefficients" is described which explains the mechanism of the behavior of living cells to these dyes.

When the protoplasm is squeezed into the sap, the pH value of the mixture is higher than that of the pure sap. The behavior of such a mixture to the dye is very much like that of the sap except that with azure B and methylene blue the rise in the pH value of such a mixture

is not so pronounced as with sap when the dye penetrates into the vacuoles.

Spectrophotometric measurements show that the dye which penetrates from methylene blue solution has a primary absorption maximum at 653 to 655 mμ (i.e., is a mixture of azure B and methylene blue, with preponderance of azure B) whether we take the sap alone or the sap plus protoplasm.

These results confirm those previously obtained with spectrophotometric measurements.

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THE REACTIONS OF CYANIDE WITH GLOBIN HEMOCHROMOGEN

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Hemochromogen and the Cyanide Compounds of Reduced Heme

Four pyrrol groups can combine to form a porphyrin. There are many different porphyrins with different side chains (Fischer, 1927). Each porphyrin can combine with iron to form a heme. The familiar hemin crystals are composed of the chloride of the particular iron-porphyrin or heme which is part of hemoglobin. Hemochromogen is defined as a pigment containing heme whose spectrum has the two characteristic bands first described by Stokes (1864).

In previous papers (Anson and Mirsky, 1925, 1928) it was shown that (1) Every hemochromogen consists of reduced heme joined to some nitrogen group; (2) Hemochromogen is always partly dissociated into and in equilibrium with its two components:

$$\text{Hemochromogen} \rightleftarrows \text{Reduced heme} + \text{Nitrogenous substance;}$$

(3) Ordinarily, nitrogen substances react with reduced heme to form only one hemochromogen-like pigment. Cyanide, however, can react with reduced heme to form two different hemochromogen-like pigments; (4) The first cyanide compound of reduced heme contains one cyanide group per heme.

These results have been confirmed and evidence further given that the second cyanide compound contains two cyanide groups per heme (Hill, 1929).

The properties of cyanide are such as to permit of the estimation of the composition of the two cyanide compounds of reduced heme. But it has not been clear what the relation is of these two compounds, both of which resemble hemochromogen spectroscopically, to an ordinary hemochromogen such as globin hemochromogen. The nitrogenous substances other than cyanide have the advantage of forming with heme only a single hemochromogen-like pigment which is undoubtedly a typical hemochromogen. But the properties of the other nitrogenous substances, as will be explained later in this paper, are such as to make difficult and uncertain the estimation of the compositions of the hemochromogens which they form from heme. As a result it has not been known whether hemochromogen in general contains one or two nitrogen groups per heme and it has not been known which of the two cyanide compounds is the typical hemochromogen, or why cyanide in particular forms two compounds with reduced heme. It was stated in the previous paper (Anson and Mirsky, 1928) that the relation of the cyanide compounds to ordinary hemochromogen was being studied by an investigation of the reactions of cyanide with heme in the presence of other nitrogenous substances.

The present experiments show that cyanide can react with globin hemochromogen in two entirely different ways. First, cyanide without displacing globin can combine with globin hemochromogen. In this reaction cyanide is *not* competing with globin for a place of attachment to the heme; it is behaving not like a typical nitrogenous hemochromogen-forming substance but probably like carbon monoxide. In addition, however, cyanide can displace globin from its combination with heme just as can ammonia and other nitrogenous substances (Anson and Mirsky, 1925, 1928). In the light of these results, the simplest although not the only explanation of the reactions of cyanide with heme in the absence of globin is that cyanide first combines with heme to form a typical hemochromogen containing one nitrogen group per heme and then combines with this cyan-hemochromogen just as it combines with globin hemochromogen.

The Biological Importance of the Hemochromogens

The biological interest in the chemistry of hemochromogen used to consist solely in its relation to the chemistry of hemoglobin which was the only heme pigment of known physiological importance. It is now clear that hemochromogens as such are of wide distribution and peculiar importance in nature. Pigments related to hemochromogen are present in aerobic tissues of plants and animals generally in concentrations great enough to permit of direct spectroscopic observation (MacMunn, 1886; Keilin, 1925, 1926; reviewed in Anson and Mirsky, 1930b). In addition the investigations of Warburg and his colleagues show that there is universally present in much smaller amount another substance related to heme and probably to hemochromogen which plays an essential rôle in the catalysis of biological oxidations (reviewed in Warburg and Negelein, 1929, and Anson and Mirsky, 1930b).

The study of the heme pigments in nature has depended entirely on a knowledge of the chemistry of pure known heme compounds *in vitro.* It is therefore important to know as much as possible about the kinds of reactions in which heme pigments can take part. In particular the ability of heme to react with nitrogen groups is significant. Aerobic cells contain a substance which can catalyze oxidations. The catalysis stops when this respiratory enzyme is combined with carbon monoxide and goes on again when the carbon monoxide is dissociated from its compound with the enzyme by light (Warburg, 1926). Similarly heme can catalyze oxidations *in vitro* and it forms with carbon monoxide a catalytically inactive compound which can be dissociated by light. But the catalytic activity of the heme so far tried is much less than that of the cellular enzyme and the sensitivity of the heme's carbon monoxide compound to light is also much less. When heme is combined with some nitrogenous hemochromogen-forming nitrogen group then the catalytic activity of heme and the sensitivity of its carbon monoxide compound to light are very much increased (Krebs, 1928). In other words, combination with a nitrogen group makes heme more like the cellular respiratory enzyme (reviewed in Warburg and Negelein, 1929, and Anson and Mirsky, 1930b).

The Reactions of Globin and Cyanide with Heme

In the present experiments different amounts of cyanide are added to reduced heme in the presence of different concentrations of globin. From the spectra of the resulting solutions it can be decided when the cyanide is competing with globin for a place on the heme molecule and when the cyanide is not competing with globin. Before describing the experiments in detail it is necessary to recall what has already been shown to happen when either globin or cyanide alone is added to reduced heme.

If denatured globin is added gradually to an alkaline solution of reduced heme the spectrum of reduced heme is gradually and finally completely replaced by that of globin hemochromogen. The addition of further globin then causes no further change in the spectrum (Anson and Mirsky, 1928). If cyanide, however, is gradually added to reduced heme there appears first a typical hemochromogen spectrum whose α band is 50 Å to the blue of that of globin hemochromogen.

But then on further addition of cyanide there appears a second two-banded spectrum similar to but definitely different from that of a typical hemochromogen. The α band is 100 Å to the *red* of that of globin hemochromogen and is much weaker than the α band of either globin hemochromogen or the first cyanide compound. This second cyanide compound obtained by adding an excess of cyanide has long been known (see spectrum in Oppenheimer's Handbuch, 1909). The first cyanide compound, which can exist only in a narrow range of low cyanide concentrations, was not observed until recently (Anson and Mirsky, 1928).

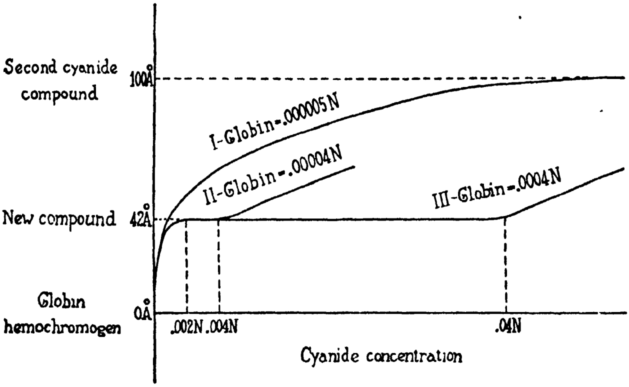


FIG. 1. Positions of α bands of pigments obtained on adding cyanide to globin hemochromogen.

The Reactions of Cyanide with Globin Hemochromogen

The new experiments are these:

1. To a dilute alkaline solution of globin hemochromogen prepared from hemoglobin (0.0084 per cent or 0.000005 N in respect to the iron of hemoglobin) there is added more and more cyanide. The α band is shifted more and more to the red, until after a shift of 100 Å it has reached the position characteristic of the second cyanide compound of reduced heme prepared by adding cyanide to heme in the absence of globin. (See Curve 1. In order to give a simple picture of all the phenomena in one graph, the graph has been made schematic and grossly inaccurate. The actual observations are given in the tables.)

2. More and more cyanide is added to globin hemochromogen in the presence of extra globin, the total globin concentration being 0.00004 N instead of 0.000005 N. The band moves to the red until when the cyanide concentration is 0.002 N, the band is 42 Å to the red of the band of globin hemochromogen. Doubling the cyanide concentration (making it 0.004 N) now causes no further change in the spectrum. But the addition of still more cyanide causes the band to move to the red again. (See Curve II.)

TABLE I

CN N	Position of α band — Å to red of 5585 Å		
	Globin = 0.0004 N	Globin = 0.00004 N	Globin = 0.000005 N
0	0	0	0
0.0001	18	18	16
0.0002	27	28	26
0.0004	33	34	33
0.001	39	40	44
0.002	41	42	49
0.004	43	43	
0.01		48	
0.02	42	57	

TABLE II

$\frac{\text{CN}}{\text{Globin}}$	Position of α band — Å to red of 5585 Å		
	Globin = 0.00008 N	Globin = 0.00004 N	Globin = 0.00002 N
0	0	0	0
125	45	43	
250	48	48	48
500	57	57	57
1,000	69	66	65
50,000			100
			No globin
			100

3. Finally, more and more cyanide is added to globin hemochromogen in the presence of a concentration of globin ten times greater still, namely 0.0004 N. Until the cyanide concentration is 0.004 N the

results are *precisely* the same as with the solutions containing ten times less globin. But whereas in the more dilute globin solution further addition of cyanide causes the band to shift to the red, in the solution 0.0004 N in respect to globin the cyanide concentration must be increased from 0.004 N to 0.04 N before the band is shifted to the red again. (See Curve III.)

4. Whenever the globin concentration is *greater* than 0.00004 N, the cyanide concentration is *less* than 0.002 N, and the band is accordingly *less* than 42 Å to the red of the band of globin hemochromogen, the position of the band depends solely on the cyanide concentration. Increasing the globin concentration has no effect on the spectrum so long as the cyanide concentration is kept constant.

5. Whenever the cyanide concentration is *greater* than 0.002 N and the band is *more* than 42 Å to the red of the band of globin hemochromogen, the position of the band depends solely on the *ratio* of the concentrations of cyanide and globin. Increasing the cyanide concentration has no effect on the spectrum provided only that the globin concentration is increased to the same extent.

6. The nature of the mixed spectrum given by a solution containing two different pigments depends on how far apart are the bands of the pigments. If the bands are far enough apart they do not fuse completely but can be distinguished separately in the mixed spectrum. For instance, the α bands of the first and second cyanide compounds of reduced heme which are 150 Å apart can both be seen in the spectrum given by a mixture of the two cyanide compounds (Anson and Mirsky, 1928). If the two bands of the two pigments are close enough together, however, they fuse to give a single band whose maximum absorption is at some wave length intermediate between the wave lengths at which the two individual pigments have their maximum absorptions. The more of one of the pigments is present the closer will the position of the intermediate band be to the band of that pigment. Thus the α bands of oxy and carbon monoxide hemoglobins (Hartridge, 1912) and of globin and ammonia hemochromogens (Anson and Mirsky, 1925, 1928) fuse to give intermediate bands whose positions are determined by the compositions of the mixtures. Finally, the bands of two pigments may not be close enough to mix to give a single intermediate band and still not far enough apart for two points of maximum

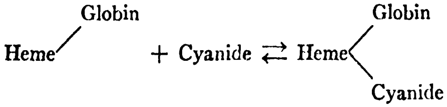
absorption to be distinguished easily in the mixed spectrum. In such cases the mixed spectrum has a band much broader than the band of a single pigment and this band becomes more and more asymmetrical as the relative concentration of one of the pigments in the mixture is increased.

In general the same mixed spectrum which is seen when one looks through a single cell containing a solution of two different pigments is also seen when one looks at the same time through two cells, one containing a solution of one pigment alone, the other containing a solution of the other pigment alone.

If one looks through two cells, one containing a solution of globin hemochromogen, the other a solution of the second cyanide compound of reduced heme, one sees a very broad asymmetrical band. It is impossible by means of optical mixtures of the spectra of globin hemochromogen and the second cyanide compound to imitate the single bands given by solutions of cyanide plus globin hemochromogen. All the spectra actually observed, however, can be imitated by means of optical mixtures of the spectra of globin hemochromogen, thesecond cyanide compound and the product with the band 42 Å to the red of the band of globin hemochromogen.

Two conclusions are drawn from the new facts:

1. Cyanide can combine with globin hemochromogen without competing with or displacing globin to form a pigment with an α band 42 Å to the red of the band of globin hemochromogen.[1] In this reaction it is not behaving as a typical nitrogenous hemochromogen-forming substance but probably like carbon monoxide.



$$\text{Heme}\diagup^{\text{Globin}} + \text{Cyanide} \rightleftarrows \text{Heme}\left\langle\begin{matrix}\text{Globin}\\ \\ \text{Cyanide}\end{matrix}\right.$$

2. Cyanide can further react with cyanide-globin hemochromogen to form the second cyanide compound of reduced heme which contains

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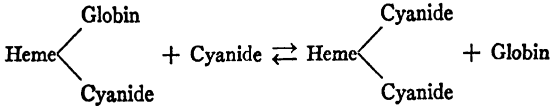
[1] Hill (1929) has observed an analogous pigment obtained by adding both nicotine and cyanide to heme. He believed that every hemochromogen contains two nitrogen groups per heme and that the compound of nicotine and cyanide with heme is a typical hemochromogen which happens to contain two different nitrogen groups.

two cyanide groups per heme. In this reaction cyanide is competing with and displacing the typical nitrogenous substance, globin.



$$\text{Heme}\left\langle\begin{matrix}\text{Globin}\\ \text{Cyanide}\end{matrix}\right. + \text{Cyanide} \rightleftarrows \text{Heme}\left\langle\begin{matrix}\text{Cyanide}\\ \text{Cyanide}\end{matrix}\right. + \text{Globin}$$

From Curve I alone one might suppose that cyanide in reacting with globin hemochromogen simply displaces the globin and forms the second cyanide compound of reduced heme. On this basis any intermediate α band observed must be the result of the fusion of the α bands of globin hemochromogen and the second cyanide compound. This theory is disproved by the fact that one cannot imitate the observed spectra by means of optical mixtures of the spectra of globin hemochromogen and the second cyanide compound. To explain the mere existence of single intermediate bands one must assume the existence of a new compound with a band between the bands of globin hemochromogen and the second cyanide compound. Since the compounds of heme with either globin or cyanide alone do not have such a band, the new compound probably contains both globin and cyanide.

Curve III shows that cyanide first reacts with globin hemochromogen to form a new compound with a band 42 Å to the red of the band of globin hemochromogen and only then reacts further with this new compound. The extent of formation of the new compound is independent of the globin concentration, so globin is not being displaced. The new compound therefore contains globin as well as cyanide, a conclusion already drawn from Curve I.

If the new compound contains globin, and if cyanide reacts with the new compound to form the second cyanide compound which does not contain globin, then this reaction must involve the displacement of globin by cyanide. In confirmation of this the extent to which the second cyanide compound is formed from the new compound of cyanide and globin hemochromogen depends solely on the cyanide to globin ratio, which is precisely what one would expect were the cyanide and globin competing for the heme.

The reason why Curve I deviates from Curves II and III even before the cyanide concentration reaches 0.002 N is now clear. If the globin concentration is low enough some globin is displaced by cyanide even if the cyanide concentration is not high enough to convert globin hemochromogen completely into cyanide-globin hemochromogen. To separate the two reactions the globin concentration must be above a certain minimum. Theoretically the two reactions can never be separated completely.

The fact that cyanide can displace globin from its combination with heme is most simply explained in the assumption that cyanide can react like a typical nitrogenous substance such as globin. This assumption, however, is not a necessary one. The affinity of hemoglobin for carbon monoxide depends on the hydrogen ion concentration; carbon monoxide and hydrogen ions compete for the hemoglobin and can displace each other. Yet these two groups do not react with hemoglobin in the same way. In all probability, carbon monoxide combines with the iron and the hydrogen ion does not, but combines with a group close to the iron and so influences the affinity of the iron for carbon monoxide.

Experimental Procedure

It remains to describe in detail the manner in which the values put down in the tables were obtained. In all cases the solutions were at 0°C. in a cold room, the final concentration of NaOH was 0.5 N, solid sodium hydrosulfite ($Na_2S_2O_4$) was added as a reducer, and the bands read after 5 minutes with a reversion spectroscope (Hartridge, 1912). In some cases the bands were read again after several hours. No change was found. The purpose of the strong alkali was to bring out the hemochromogen-forming capacity of globin (Anson and Mirsky, 1928).

The three solutions of Table I were 0.000005 N, 0.00004 N and 0.0004 N in respect to globin. The 0.000005 N solution was prepared by adding 2.5 ml. of 0.2 N HCl to 1 ml. of 8.5 per cent horse carbon monoxide hemoglobin and then diluting with NaOH. This solution was accordingly 0.000005 N in respect to globin, heme and iron. The 0.00004 N and 0.0004 N solutions contained 0.00001 N globin and heme from hemoglobin and in addition denatured globin prepared by the oxalic acid-acetone method (Anson and Mirsky, 1930a).

The 0.00008 N solution of Table II contained 0.00002 N globin and heme from hemoglobin and in addition 0.00004 N globin prepared by the acetone method. To obtain the 0.00004 N and 0.00002 N solutions, the 0.00008 N solution was diluted with 0.5 N NaOH.

The band 42 Å to the red of the α band of globin hemochromogen is a sharp band intermediate in intensity as well as in position between the α bands of globin hemochromogen and the second cyanide compound of reduced heme.

When the cyanide to globin ratio reaches 1,000 the band begins to be too asymmetrical to be read with the reversion spectroscope.

Alkaline solutions of hydrosulfite attack the cements which were tried, including de Khotinsky cement. The decomposition products of the cements influence the spectra. This difficulty was avoided by using cells made of paraffin blocks and pieces of glass microscope slides. Such cells can be made in a few minutes. From paraffin blocks 1.5 cm. or 3 cm. thick depressions are melted out with a hot iron. These depressions are closed on the sides of the blocks by melting in pieces of glass. To make a double cell to permit looking through two solutions at once, a piece of glass which is to act as a partition is first melted between two blocks of paraffin. If cells only a few millimeters thick are desired, it is better to place slightly softened but not melted paraffin between two pieces of glass which are then pressed together in a vise until they are the desired distance apart. The glass sides of the cell are thus automatically made parallel. Finally some of the paraffin is melted out to form the cell.

The Nature of the Two Cyanide Compounds of Reduced Heme

A priori there are four possibilities in regard to the nature of the two cyanide compounds of reduced heme. Neither of the two cyanide groups may be a typical nitrogenous hemochromogen-forming group; both may be; cyanide may be acting as a typical nitrogenous group in forming only the first; or in forming only the second compound. The present experiments do not decide conclusively which of these four possibilities corresponds to the truth. What has been made clear is that cyanide can combine with heme in two different ways. In one reaction it does not compete with or displace globin; in the other it does. In the light of these results and of the fact that the spectrum of the first compound is similar to that of globin hemochromogen and the spectrum of the second compound is similar to that of cyanide globin hemochromogen, the simplest theory is that the first compound is a typical hemochromogen and the second compound a compound of cyanide with cyanide hemochromogen.

The Composition of Hemochromogen

If the first cyanide compound of reduced heme which has one cyanide group per heme is a typical hemochromogen, then it is probable that hemochromogens in general contain one nitrogen group per heme. It would obviously be desirable to confirm or refute this conclusion by independent investigation of the composition of some hemochromogen whose nitrogenous substance forms only one hemochromogen-like compound with heme. The attempts to do this have not yet led to any conclusive results. Von Zeynek found by analyses of the precipitates that ammonia hemochromogen (1898) contains one nitrogenous group and pyridine hemochromogen (1910) two nitrogenous groups per heme. But such direct analyses must remain of dubious significance until it is shown that in the preparation of the precipitates no hemochromogen-forming nitrogen groups are split off from the heme and no non-hemochromogen-forming nitrogen groups added to the carboxyl groups.

Two molecules of pyridine must be added to each molecule of heme in order to convert heme into pyridine hemochromogen (R. Hill, 1926, 1929). If all the pyridine added were combined with the heme this would mean that pyridine hemochromogen contains two pyridines per heme. But some of the pyridine added must be free to drive the equilibrium:

$$\text{Pyridine hemochromogen} \rightleftarrows \text{Heme} + \text{Pyridine}$$

to the left. It cannot be decided from Hill's experiments whether or not this free pyridine is negligible. In dilute solution of the pigment the concentration of free pyridine needed to prevent visible dissociation of the hemochromogen may be determined. It is not known whether the same concentration of free pyridine is needed to prevent visible dissociation of the hemochromogen in the concentrated solutions actually used. As has already been pointed out (Mirsky and Anson, 1929) the equilibrium is complicated by the aggregation and precipitation of both pyridine hemochromogen and reduced heme:

$$\begin{array}{ccc}\text{Pyridine hemochromogen} & \rightleftarrows & \text{Reduced heme} + \text{Pyridine}\\ \downarrow & & \downarrow\end{array}$$

Precipitation or aggregation of the hemochromogen would drive the equilibrium to the left and so reduce the concentration of free pyridine needed to prevent detectable dissociation of the hemochromogen. Precipitation of the heme would have the opposite result. It is not known what effect concentration has on the relative velocities and extents of the aggregation of the two pigments.

Dr. Northrop has suggested that it might be possible to estimate the free pyridine in a solution of pyridine hemochromogen by shaking the aqueous solution with a solvent which does not mix with water but in which pyridine is soluble, estimating the total pyridine in this solvent, and then calculating the free pyridine in the water from the distribution constant.

Cyanide and Carbylamine

Warburg, Negelein and Christian (1929) have recently described some reactions of carbylamine with hemoglobin which in some ways resemble the reactions of cyanide with heme. Carbylamine can combine with hemoglobin in two different ways. In one reaction it competes with carbon monoxide, in the other it does not. Native globin in hemoglobin is not behaving like a typical nitrogenous substance such as ammonia or denatured globin. Whether one of the carbylamines is behaving like a typical nitrogenous substance has not yet been determined.

Conclusions

Cyanide can react with globin hemochromogen in two different ways. In the first reaction cyanide combines with globin hemochromogen without displacing or competing with globin. In the second reaction cyanide displaces globin.

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THE ACCUMULATION OF ELECTROLYTES

I. The Entrance of Ammonia into Valonia macrophysa

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(Accepted for publication, June 25, 1930)

Experiments on the penetration of NH_3 into *Valonia macrophysa*, carried out in Bermuda in 1924–27, are described in the present paper. The interpretation of these results will receive further discussion in subsequent articles.

In order to ensure uniformity of material and of sea water a large number of cells, collected at the same time, were kept under the same conditions and all the sea water used for these cells and for the experiments was collected at the start and kept in carboys. The average volume of the cells was about 0.5 cc.

The experiments were carried out in stoppered bottles each containing 2400 cc. of solution with about 50 cells in each bottle. When the bottles were placed in the ocean they were fastened beneath a specially constructed raft so that the light received was mostly reflected from the whitish coral sand of the bottom. In the experiments in the laboratory direct sunlight was avoided. Growth and absorption of ammonia were more rapid in the ocean.

The temperature in the ocean varied from 20° to 25°C., but in the course of any one experiment the variation was not more than 2° or 3°C., except in those lasting several weeks. In experiments carried on in the laboratory the variation was much greater.

The NH_4Cl was added to the sea water in the form of a solution, 0.6 molar or normal in NH_4Cl; the pH value of the sea water was adjusted by the addition of HCl or NaOH. After exposure to the sea water containing NH_4Cl, the sap was collected by means of a pipette having a sharp point which was thrust into the cell (in order to avoid loss of NH_3 by suction, the sap was forced into the pipette with the fingers). Care was taken in collecting the sap to use some large and some small cells so that the sap[1] could be considered as taken from an average cell of about 0.5 cc. When sufficient sap had been extracted, it was blown out into a small beaker and covered. Two 5-cc. samples (for zero point determinations there were three) were pipetted out for sodium-potassium determinations, two 1-cc. samples for ammonia, two 1-cc. samples for halide, and a single 1-cc. sample for pH and sulfate. The samples for sodium-potassium analysis were stored in quartz, the others in pyrex vessels. The samples for ammonia determination were at once acidified to prevent escape of NH_3.

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[1] For analysis of the sap see Osterhout, W. J. V., *J. Gen. Physiol.*, 1922-23, 5, 225.

Analysis of the various samples was made according to standard procedures: potassium was determined as potassium perchlorate (accurate with quartz vessels, to within 0.1 per cent): sodium by indirect analysis as sodium chloride (accurate to within 1 per cent); ammonia (total) by colorimetric determination as $NHg_2I \cdot H_2O$ after distillation of the ammonia from the strongly alkaline solution and subsequent Nesslerization (distillation was necessary because the turbid condition of the Nesslerized raw sap made color estimation exceedingly difficult); halide was determined by the Volhard method; the pH values were determined colorimetrically, allowing for the salt error by adding NaCl to the standard buffer solutions.

For the volume measurements, each cell was carefully dried with cloth and filter paper and weighed rapidly to milligrams, then placed in 0.005 M NH_4Cl sea water in a specific gravity bottle which was then immersed in a thermostat and allowed to come to a temperature of 25°C. The bottle was then dried and weighed to milligrams. From these weighings the cell volume was calculated. Such cells were photographed in groups (under water) to facilitate future identification, the volume of each being noted on the photograph.

When NH_4Cl is added to the sea water it soon appears inside the cell,[2] where its concentration may become over 40 times as great as the sea water, thereby lessening the specific gravity of the sap[3] so much that the cells, leaving their normal situation on the bottom, rise to the surface, where they continue to grow. The change in the specific gravity of the sap is shown in Fig. 1.[4]

Experiments were made to determine the rate of penetration at various pH values. Since the initial rate is most easily interpreted the experiments were limited to 10 minutes which is as short a period as is practicable.

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[2] For experiments on the penetration of NH_3 into *Valonia* see Brooks, M. M., *Pub. Health Reports*, Washington, D. C., 1923, **38**, 2074; for experiments on other cells see Warburg, O., *Z. physiol. Chem.*, 1920, **66**, 305; Harvey, E. N., Carnegie Institution Publication, 1914, *No.* **183**, p. 131; Irwin, M., *J. Gen. Physiol.*, 1925–26, **9**, 235; Jacobs, M. H., Harvey Lectures, 1926–27, p. 146.

[3] *Cf.* Cooper, W. C., Jr., Dorcas, M. J., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1928–29, **12**, 427. Osterhout, W. J. V., Some fundamental problems of cellular physiology, New Haven, 1927. This curve is based on experiments in the laboratory.

[4] The change is more rapid when the cells, enclosed in bottles, are placed in the ocean; this may be due in part to light (*cf.* Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1923–24, **6**, 47; Hoagland, D. R., Hibbard, P. L., and Davis, A. R., *J. Gen. Physiol.*, 1926–27, **10**, 121.

In each bottle containing 120 cc. of solution were placed 6 cells (each cell containing about 0.2 cc. of sap). Cells were selected as far as possible of the same size and shape. The bottles were placed on a slowly revolving wheel to keep the contents stirred. Ordinarily this treatment produced no injury which could be detected during the experiment or by subsequent observation (during several days) of cells kept in sea water. The ammonia (*i.e.*, $NH_3 + NH_4OH + NH_4^+$) of the sap of untreated cells (about 0.0006 M) was subtracted from the ammonia values of the sap of treated cells to give the values shown in Fig. 2. The pH value of the sea water was adjusted by adding NaOH or HCl; the total ammonia of the sea water was kept constant.

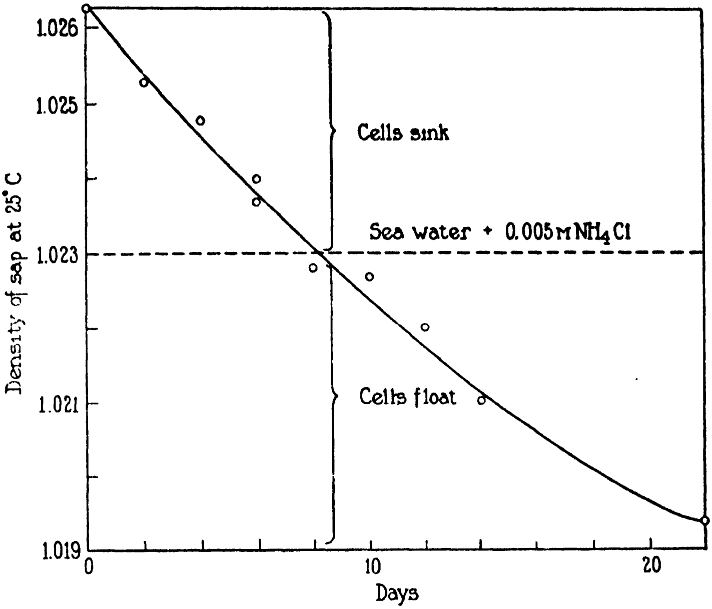

FIG. 1. Time curves showing the decrease in the specific gravity of the sap during the penetration of ammonia from sea water (at pH 8.3) containing 0.005 M NH_4Cl.

The results are shown in Fig. 2 and it is evident that as the concentration of the undissociated base (*i.e.*, $NH_3 + NH_4OH$) increases the rate[5] of penetration of ammonia (total) also increases. But it is not easy to determine just how closely they agree. If we knew the rate of penetration when the total ammonia (*i.e.*, $NH_3 + NH_4OH + NH_4^+$) is present as the undissociated base ($NH_3 + NH_4OH$) it would be easy to see whether the rate is reduced to one half when the concentration of the undissociated base is reduced to one half, but unfortunately we cannot safely perform experiments at a sufficiently high pH value to ensure that practically all of the ammonia is present in undissociated form (even at pH 10.1, which is as high as we have gone, less

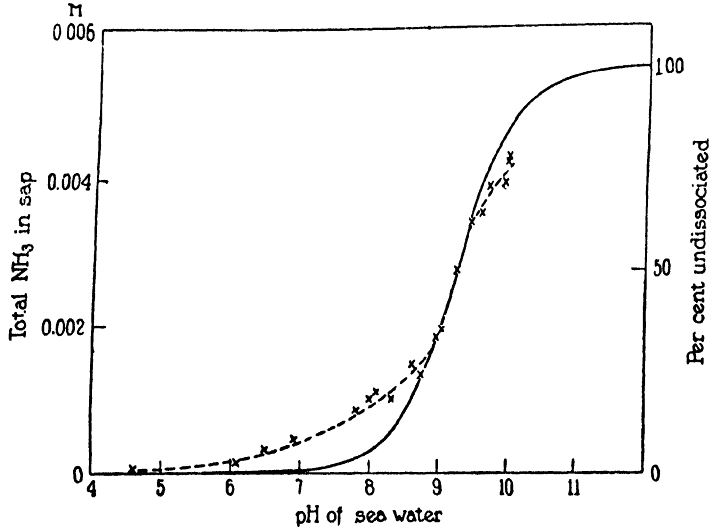

FIG. 2. Shows the increase in concentration of total ammonia in the sap after an exposure of 10 minutes (at 21° ± 1°C.) to sea water containing 0.01 M NH_4Cl at various pH values. Broken line, observed values; continuous line, calculated curve for dissociation of $NH_4OH + NH_3$ in distilled water $\left(\frac{(NH_4^+)\,(OH^-)}{(NH_4OH) + (NH_3)} = K_B = 10^{-4.74}\right)$. Since it happens that the inflection points of both curves lie at approximately pH 9.26 they have been made to coincide there: at that point the ordinate of the observed curve is 0.00275 M and this point is taken as 50 per cent of the calculated dissociation curve (whose inflection point and 50 per cent dissociation point coincide).

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[5] Experiments with dead cells show little or no effect of pH value on penetration.

than 87 per cent of the total ammonia is regarded as present as the undissociated base).

It seems probable that in the absence of complicating factors the curve might resemble the equilibrium curves[6] for H_2S and CO_2, so that the inflection point would occur where the rate of entrance and the per cent of undissociated base was one half the maximum. We find that the point of inflection of the rate curve occurs at pH 9.25, which corresponds fairly well to the point (9.26) at which one half the total ammonia is in the form of the undissociated base ($NH_3 + NH_4OH$) as calculated from conductivity data of solutions of ammonia in distilled water.[7] This would suggest that the initial rate of entrance reaches 50 per cent of its maximum when the undissociated base reaches 50 per cent of its maximum concentration.

In order to compare the two curves we have made them coincide at their inflection points as shown in Fig. 2.

We should expect the point of inflection of the dissociation curve to occur at a somewhat higher pH value in sea water on account of the greater ionic strength. In order to determine approximately this value for sea water NH_3 was circulated in a closed glass vessel (designed by Dr. Grinnell Jones[8] to whom our thanks are due for allowing us to use his invention in advance of publication), containing on one side ammoniacal sea water and on the other, distilled water to which sufficient NaOH had been added to bring the pH value above 12 under which conditions all of the ammonia exists as the undissociated base ($NH_3 + NH_4OH$). The total ammonia was kept constant and the pH value of the sea water was varied by adding HCl or NaOH; after equilibrium had been attained at any given pH value a sample was taken of the ammoniacal water in equilibrium with the ammoniacal sea water and the ammonia determined by colorimetric comparison. We therefore made measurements which gave a curve indicating that the inflection point, *i.e.*, the point where half the total ammonia is in the form of the undissociated base, is in the neighborhood of pH 9.5.

If the rate of entrance of ammonia is proportional to the external concentration of the undissociated base we might expect the curves to coincide, but this could be true only if the internal pH value remained constant. If, for example, the pH of the sap were kept constant at 12 so that the entering ammonia did not dissociate inside, there would be no complications, but with sap at lower pH values a part of the entering NH_3 would be transformed to ions and, therefore, the back pressure, or the tendency of the undissociated base to return to the sea water, would be diminished. In other words, the rate of penetration of ammonia would be a function not only of the external pH but of the internal pH as well.[9]

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[6] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1925-28, **8**, 131; Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925-26, **9**, 255.

[7] This gives $K_A = 10^{-14} - K_B = 9.26$ and $K_B = 4.74$.

[8] Jones, G., *J. Am. Chem. Soc.*, 1928, **50**, 1600.

Our experiments show that the entrance of NH_3 raises the internal pH value rapidly (Fig. 3), this effect being greater the higher the concentration of undissociated ammonia in the sea water. Hence we expect the rate to be relatively higher at the lower pH values of the sea water because under these conditions there is a smaller penetration of ammonia and consequently less effect on the pH of the sap (normally about 5.8). This would increase the ordinates of the curve progressively toward the left[10] and consequently make the slope of the curve less steep, *i.e.*, like that of the observed curve.[11]

The fact that the initial rate of penetration increases when the concentration of undissociated molecules increases suggests that it is chiefly undissociated molecules which penetrate, but it has been pointed out elsewhere that this does not necessarily follow.[12] It does indicate that the penetrating substance is NH_3 or NH_4OH or the ion pair $NH_4^+ + OH$, rather than NH_4Cl whose rate of entrance would not be expected to increase as the external pH value increases.

That the substance penetrating at the start is largely NH_3 (or

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[9] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13,** 261.

[10] We should expect a similar effect if penetration depended on chemical combination of undissociated molecules with some constituent of the protoplasm (or on absorption), since the amount of the compound formed would determine the rate of penetration and this would increase more and more slowly as the external concentration of undissociated molecules increased.

[11] This can easily be seen by making the ordinates of the dissociation curve progressively larger as we go toward the left and then multiplying all the ordinates by the same factor so as to make the maximum ordinate the same as for the dissociation curve.

[12] This results from the fact that if the ion pair $NH_4^+ + OH^-$ enters, its penetration is proportional to the ionic activity product $({}^aNH_4) \times ({}^aOH) = K\,({}^aNH_4OH)$, so that as the penetration of the indissociated base increases that of the ion pair will also increase. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1929–30, **13,** 261.

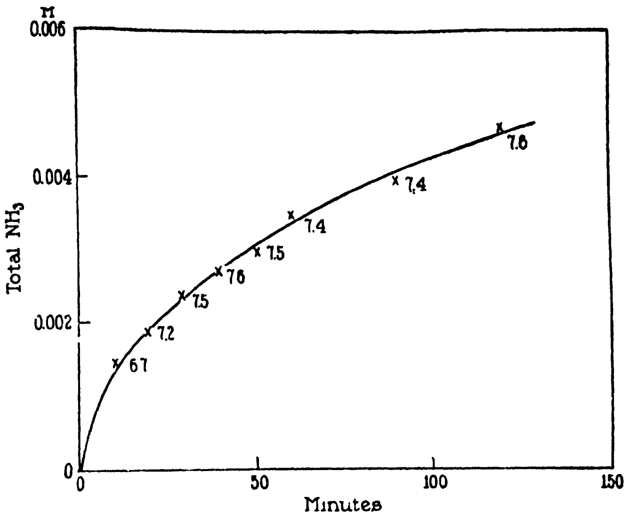

FIG. 3. Time curves showing the increase of pH value of sap during penetration of ammonia from sea water, at pH 8.1, containing 0.005 M NH_4Cl.

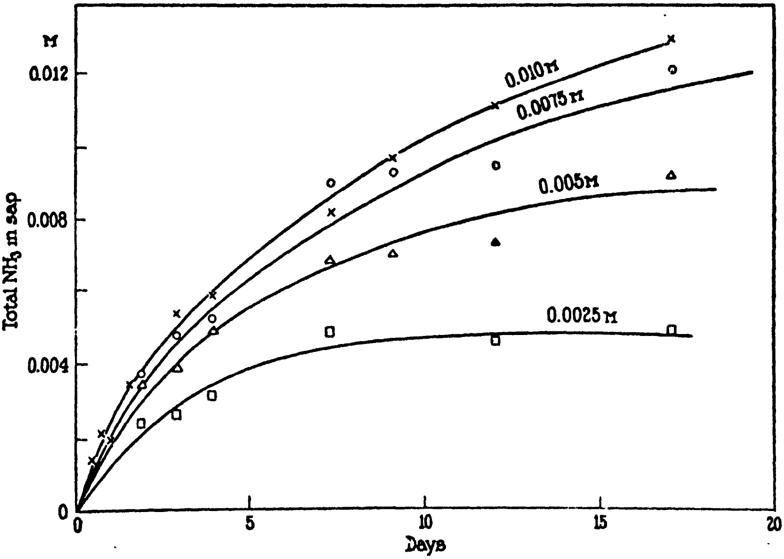

FIG. 4. Time curves showing increase of total ammonia during penetration from sea water (at pH 8.3) containing various amounts of NH_4Cl.

NH_4OH) rather than NH_4Cl is rendered quite certain by the data given in Fig. 3, showing the rise in pH value as the total NH_3 in the sap increases (for it is evident that the penetration of NH_4Cl would not produce such an effect). The pH value of the sap soon reaches a maximum[13] and may subsequently fall off in a gradual manner.

As has been said already, the increase of undissociated ammonia does not raise the rate of penetration of ammonia as much as would be expected if there were no change in the internal pH value. The time curves in Fig. 4 bear out this statement. These curves do not reach a true equilibrium, for the cell keeps on growing; furthermore, when the concentration of NH_3 in the sap reaches a certain point (less than 0.3 M) injury causes it to come out.[14]

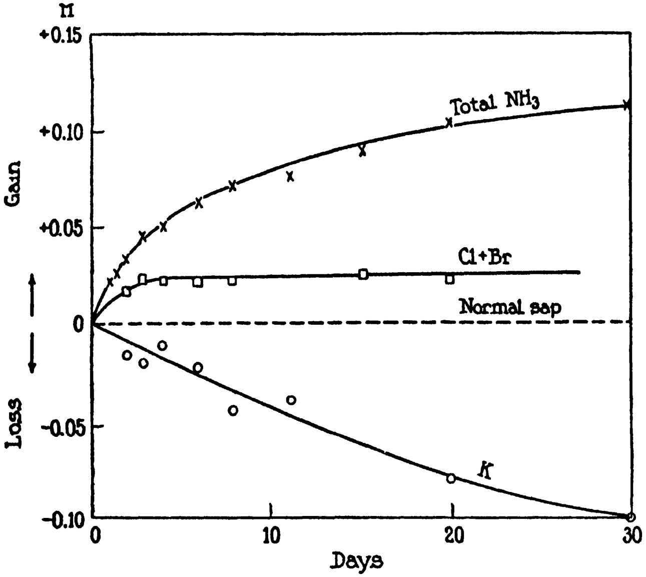

FIG. 5. Time curves showing increase in concentration of total ammonia and of halide, and decrease in the concentration of potassium during the penetration of ammonia from sea water (at pH 8.3) containing 0.005 M NH_4Cl.

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[13] With 0.001 M NH_4Cl in the sea water this is 6.6, with 0.01 M NH_4Cl it is 8.2, the pH value of the sea water being 8.0.

[14] *Cf.* Irwin, M., *J. Gen. Physiol.*, 1925-26, 9, 235.

Fig. 5 shows that as the total ammonia in the sap increases, the concentration of potassium in the sap falls off, and measurements of volume[15] indicate that the decrease was not due merely to the absorption of water but that some potassium actually diffused out of the cell. The behavior of Na is not yet certain: further experimentation is in progress.

Fig. 5 also shows that the initial entrance of ammonia is accompanied by an increase in the concentration of halide, which soon reaches a fairly constant value dependent on the external pH. In the absence of ammonia in the sea water, there is no such variation in the halide of the sap with change in the external pH.

It may be added that the growth of the cells is increased by the addition of NH_4Cl to the sea water.

SUMMARY

When 0.005 M NH_4Cl is added to sea water containing cells of *Valonia macrophysa* ammonia soon appears in the sap and may reach a concentration inside over 40 times as great as outside. It appears to enter as undissociated NH_3 (or NH_4OH) and tends to reach a pseudoequilibrium in which the activity of undissociated NH_3 (or NH_4OH) is the same inside and outside.

When ammonia first enters, the pH value of the sap rapidly rises but it soon reaches a maximum and subsequently falls off. At the same time there is an increase of halide in the sap which, however, does not run a parallel course to the ammonia accumulation, but it comes to a new equilibrium value and remains constant.

The increase in NH_3 in the sap is accompanied by a decrease in the concentration of K.

As NH_3 enters the specific gravity of the sap decreases and the cells rise to the surface and continue to grow as floating organisms. The growth of the cells is increased.

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[15] The cells grow faster when NH_3 is added to the sea water.

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THE VARIATION OF ELECTRICAL RESISTANCE WITH APPLIED POTENTIAL

II. THIN COLLODION FILMS

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(Accepted for publication, June 12, 1930)

I

Analogies to certain physiological phenomena have been observed in the behavior of collodion membranes. Collander,[1] Michaelis,[2] and Northrop[3] have reported resemblances in the penetration of certain substances; Michaelis[4] has shown the similarity of the potential differences of such membranes to those of apple skin and other cuticle; Labes and Zain[5] have imitated with collodion tubes such bioelectric effects as current of injury, polarizations in direct current, and the phenomena of electrotonus.

Furthering these analogies, the present paper amplifies some observations previously made[6] on very thin collodion membranes, which resemble the protoplasm of *Valonia* cells under some conditions[7] in having an apparent resistance to direct current which varies with the voltage and the direction of an applied potential.

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[1] Collander, R., *Soc. sci. Fennica, comment. biol.*, 1926, **2**, 1; *Kolloidchem.Beih.*, 1924, **19**, 72.

[2] Michaelis, L., and Weech, A. A., *J. Gen. Physiol.*, 1928-29, **12**, 55. See also Fujita, A., *Biochem. Z.*, 1926, **170**, 18.

[3] Northrop, J. H., *J. Gen. Physiol.*, 1927-28, **11**, 233; 1928-29, **12**, 435.

[4] Michaelis, L., *J. Gen. Physiol.*, 1925-28, **8**, 33; *Bull. National Res. Council*, 1929, No. 69, 119.

[5] Labes, R., and Zain, H., *Arch. exp. Path. u. Pharm.*, 1927, **125**, 29, 53; **126**, 284, 352.

[6] Blinks, L. R., *Proc. Soc. Exp. Biol. and Med.*, 1928-29, **26**, 359. Shortly after the publication of this preliminary note, Ebbecke (Physiological Congress, Boston, 1929) described similar effects with collodion, stating that the current across a membrane from HCl to salt was greater than from salt to HCl.

[7] Blinks, L. R., *J. Gen. Physiol.*, 1929-30, **13**, 793.

The conductance of collodion membranes was found by Michaelis[8] to vary greatly and characteristically with the solution of electrolyte in contact with them. His membranes, thoroughly imbibed by long washing, were usually in contact with the same solution on both sides. When they separated two different solutions (as in the experiments[9] where the transfer number of ions in the membrane was determined) there was at first a change of resistance with time, necessitating the adjustment of the current by a ballast resistance. This change was ascribed chiefly to polarization at the platinum electrodes.

In the present experiments, the most marked effects were found when the membranes separated two different solutions of different conductivity, such as HCl and KCl. The resistance then depended upon which ions were carried across the membrane, and the potential gradient under which they moved. It is believed that the changes are those of real ohmic resistance and that they present no phenomena requiring any special properties of collodion to explain, since the effects may be seen in other systems. The collodion film is thus used merely to obtain a very short conducting system separating two different solutions.

A rise of resistance always occurs when ions of lower mobility are moved into a conductor and supplant those of higher mobility. Devices for maintaining a constant current in a tube of electrolyte under these conditions are used in the determination of transport number by the "moving boundary" method.[10] A similar effect is more strikingly seen when a current passes through a fine capillary, filled with KCl solution and dipping into sea water; the resistance then rises when the positive current passes inward, and falls, when, it passes outward. This is an effect which must be controlled in the experiments performed with cells impaled on capillaries.[11]

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[8] Michaelis, L., Green, A. A., and Weech, A. A., *J. Gen. Physiol.*, 1928–29, **12**, 473.

[9] Michaelis, L., Weech, A. A., and Yamatori, A., *J. Gen. Physiol.*, 1926–27, **10**, 685.

[10] Longsworth, L. G., and MacInnes, D. A., *J. Opt. Soc. Am. and Rev. Sci. Instr.*, 1929, **19**, 50.

[11] Blinks, L. R., *J. Gen. Physiol.*, 1930–31, **14**, 139.

In both the above cases the limits are represented by the resistance of the conductor when totally filled with one or the other solution. They tend to be reached at all current densities, the rise being slower with small voltages than with large. When sharp boundaries are maintained, this must be strictly true. But under very small potential gradients the boundaries become less sharp, and backward diffusion will prevent the complete substitution of one solution for another. (A heating effect may further contribute to this diffuse boundary at very high current densities.)

The most satisfactory explanation for the apparent equilibrium at any given potential in these collodion membranes is that a boundary of greater or less sharpness is formed, composed of different proportions of the ions involved. This maintains itself under the two opposed gradients of potential and diffusion, and as long as these remain constant, the character and position of the boundary is unchanged, giving a constant resistance. When either is changed the resistance changes. The process is exaggerated by the extreme thinness of the membrane, so that the boundary has but to move a short distance to the right or left in order to completely fill the membrane with one or the other electrolyte.

II

The membranes were prepared from ordinary Merck's collodion U. S. P.; or from Parlodion dissolved in 75 per cent alcohol, 25 per cent ether. The kind of collodion seemed to be immaterial for these effects. A small amount of the solution was poured upon a clean glass plate and another plate gently placed over it (avoiding bubbles), spreading the collodion into a film. The plates were then carefully slid apart and stood on end to dry. 15 minutes' drying was often sufficient for these very thin films, but usually they were allowed a day or more. They adhere very tightly to the glass and may be stored in this condition. Usually, however, they were removed from the glass and stored spread out on filter paper. This was done by immersing the glass plates in water until the films were free (loosened, if necessary, by a camel's hair brush) and floated to the top. They were then spread into a smooth sheet, and lifted out, supported on filter paper inserted from below. Dried flat they now remained slightly attached to the paper, and could be stored in folders until needed.

From regions which showed brilliant red and green interference colors 2 cm. squares of paper and film were cut with shears and the film cemented over the ends of tubes for use. These tubes were usually of the Y-form shown in Fig. 1. One arm of the Y was gently heated and the end ringed with the low melting point wax "Picein" which adheres well to both glass and collodion. While still warm it was brought gently in contact with the square of collodion which formed a firm seal to the glass and came free of the filter paper. The other arm of the Y was filled with a plug of stiff agar gel, imbibed with the solution used in the Y-tube. The Y-tube was usually filled for a distance of about 1 cm. up the vertical neck. For emptying it was inverted and drained. Great care was needed to avoid vibration at all times as the thin membranes were easily broken by jar, even with the small head of liquid used.

The arms of the Y-tube dipped into intermediate vessels filled with the desired solutions as shown. The electrodes were large ones of chloride-coated amalgamated lead, and were practically free of polarization at any current density used in these experiments. No resistance changes of great magnitude were observed in the system of conductors leading up to the membranes. With both arms of the Y-tube plugged with agar the resistance of the system under various conditions was between 500 and 1500 ohms; it remained constant to 50 or 100 ohms during long current flows under applied potentials of as much as 4 volts. This value formed the base which was subtracted from the total resistance to give that of the membrane. The resistance was measured in the direct current bridge previously described.[12]

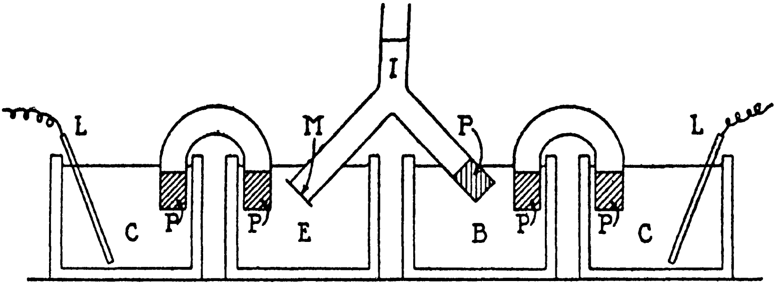

FIG. 1. Diagram of apparatus for measuring resistance of collodion films. The membrane (*M*) is cemented over one arm of the Y-tube, and separates the inner solution (*I*) from the outer solution (*E*). The other arm, plugged with agar (*P*), dips into the cup (*B*). U-tubes, plugged with agar (*P*), connect with the cups (*C*) into which dip the electrodes (*L*).

The membranes introduced resistances from a few hundred ohms (when there were large leaks) to several megohms, as measured under standard conditions (in contact with 1.0 M KCl solutions). Neither of these extremes showed the phenomena of variable resistance as strikingly as the membranes of intermediate values most of which, as used, ranged between 3000 and 50,000 ohms, under standard conditions. With care the solutions in contact with the membrane could be changed 8 or 10 times, and a return made to the first resistance value when in contact with the initial solutions. Sometimes a jar or the passage of a large current itself suddently reduced the value of resistance under all conditions to a new level, at which the effects still took place, although often with a changed percentage. The resistance of the membranes had therefore to be checked frequently under standard conditions (in contact with 1 M KCl). When this was done a considerable series of comparisons could be instituted.

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[12] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13**, 361.

III

When a small potential was applied to a membrane separating two solutions, a definite resistance was soon reached, at which the bridge maintained a constant balance. When the potential was changed, a new value was produced, either higher or lower, depending upon the direction and intensity of the potential gradient: 15 seconds was usually sufficient to reach this new constant level, and this time was chosen as a standard in which to make the balance. In some cases a longer time was required, but in general the values at 15 seconds represented fully 95 per cent of the steady values.

The largest absolute changes of resistance were usually found in freshly made membranes immediately after being sealed to the tubes. As an example, such a new membrane had a net resistance of 9500 ohms when separating a solution of normal HCl inside the tube from sea water outside. This was the value when 50 mv. was applied to it, in such a direction that the positive current passed outward (from HCl toward sea water), moving H ions across the membrane. When the potential was increased in this direction the resistance fell further, reaching at 100 mv. 7800 ohms, and at 0.5 volt a value of 5200 ohms. At higher voltages the resistance became nearly constant at about 4800 ohms. On the return to a lower applied potential the resistance rose again and at 100 mv. was 7700 ohms. The potential was now reversed so that the positive current passed from sea water inward to HCl. At 50 mv. the resistance was 15,000 ohms; at increased voltages it rose rapidly, reaching at 0.5 volt 18,300 ohms, and at 3.3 volts 21,500 ohms. On the return to 100 mv. the value was 15,600 ohms.

After this membrane had been placed in sea water for 2 days, the resistance rose only to about 15,500 ohms at the maximum inward potential. A day later, after many measurements had been made, the range was from 500 ohms (current outward) to 5800 ohms (current inward). There had evidently been progressive injury which increased the absolute permeability to ions. But the *percentage* of resistance rise under the same conditions had increased, probably because the current densities in the earlier experiments had not been sufficient for sweeping out all H ions from the membrane.

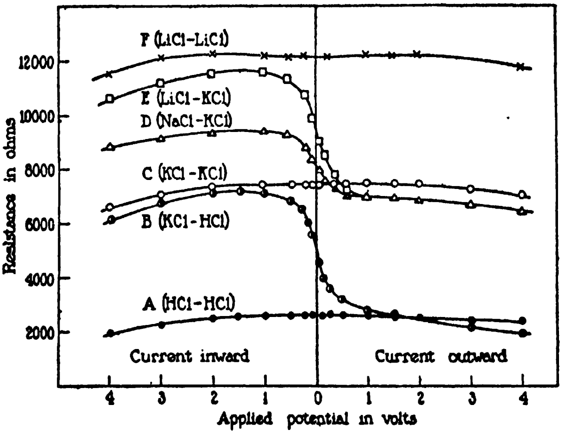

FIG. 2. Variation of resistance with applied potential in a membrane separating molar solutions, as follows:

Curve	*Outside solution*	*Inside solution*
A	HCl	HCl
B	KCl	HCl
C	KCl	KCl
D	NaCl	KCl
E	LiCl	KCl
F	LiCl	LiCl

Potentials increasing to the left of zero cause a flow of positive current *inward;* to the right *outward.*

This experiment may be considered analogous to many biological cases, where a cell encloses sap of high electrolyte concentration and is surrounded by water of low electrolyte concentration.

Even greater rises of resistance may be produced by greater dilutions of the sea water outside the membrane. But more useful information was gained by keeping the concentrations equal on both sides of the membrane, and varying the *composition* of the solutions. In most of the experiments the concentration was kept at 1.0 molar, and chlorides only were employed, the cations being varied. Such a group of experiments is shown in Fig. 2 for HCl, KCl, NaCl, and LiCl. It is seen that the resistance increases in the order given. When two salts were separated by the membrane the resistance rose when the direction of the current was such as to carry the less mobile ions across the membrane. In general the highest value then reached (at high potentials) was a little lower than that found (at all potentials) when the membrane was exposed to the more poorly conducting solution on both sides. This showed that there was still some mixing of ions in the membrane at the highest potentials. The resistances of the membranes, when wholly imbibed with the given solutions, were about in the proportions of the specific resistances of aqueous solutions of the equivalent concentration. The membranes thus did not show any specific properties in exaggerating differences in ionic mobilities, such

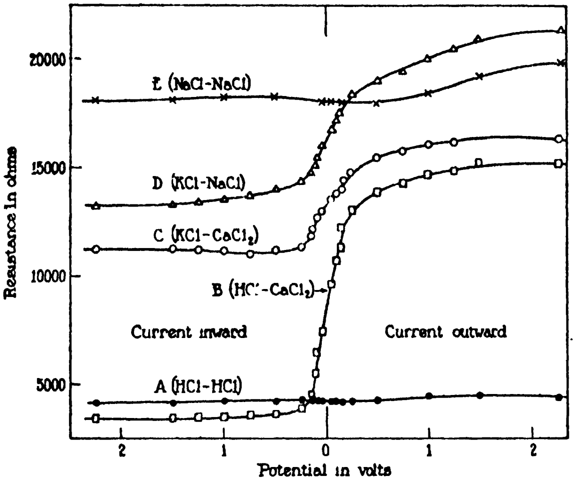

FIG. 3. Variation of resistance with applied potential in a membrane separating molar solutions of various kinds: Curve *A*, HCl both sides; *B*, HCl outside, CaCl$_2$ inside; *C*, KCl outside, CaCl$_2$ inside; *D*, KCl outside, NaCl inside; *E*, NaCl inside and outside. The membrane had evidently been somewhat injured between Curves *A* and *B*, and between *D* and *E*. Potentials increasing to left of zero cause inward current, to right, outward.

as Michaelis found in his celloidin (Schering) membranes. The different kind of collodion, the higher salt concentrations used, and the thinness of the membranes may all account for the absence of this effect here. The membrane in 1 M LiCl had about twice the resistance it had in 1 M KCl, and in the latter had about three times that in 1 M HCl. The value with NaCl fell between KCl and LiCl, while in 1 M $CaCl_2$ (Fig. 3) it had a slightly lower resistance than in 1 M NaCl. These are roughly proportional to the specific resistances of the solutions.

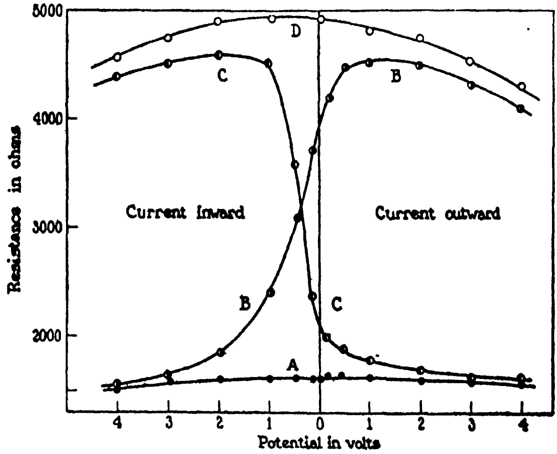

FIG. 4. Variation of resistance with applied potential in a membrane separating molar solutions of HCl and KCl. Curve *A*, HCl on both sides; *B*, HCl outside, KCl inside; *C*, KCl outside, HCl inside; *D*, KCl on both sides. Potentials increasing to left of zero cause inward current, to right, outward.

The alternating current resistance of these membranes was usually equal to that of direct current just where the curves crossed the line of zero potential.

While the resistances are in general dependent on the solutions with which the membranes are in contact, and hence are reversible when the solutions are changed side for side, there are also slight, and sometimes very large, asymmetries, which greatly distort the curves. Fig. 4 shows the values reached when KCl and HCl were applied to a membrane in four different combinations. With HCl inside the tube and KCl outside the curve crosses the zero line at quite a different point from where it does with the opposite arrangement. The two curves also cross each other at a point about 300 mv. from the zero line.

It is also to be noted that the curve D is distinctly rounded, with the highest resistances at the lowest potentials. This was often found to be the case in the membranes, and may be due to a widening of current paths under high potentials. But the opposite shape, with the resistance *rising* on both sides of zero was occasionally found, and is so far without explanation. Fig. 5 shows a case of this kind, and also illustrates two other anomalies. It is evident that the resistance rise is not symmetrical about zero potential, being mostly confined to an *inward* current, decreasing at first with *outward* currents, and finally

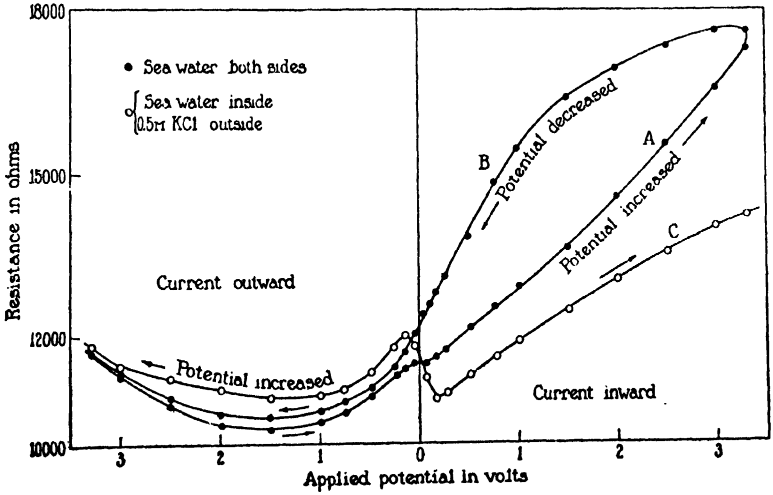

FIG. 5. Anomalies in the variation of resistance with potential. Curves *A* and *B* are for a membrane with sea water on both sides. Curve *A:* potentials decreased from left to zero, then reversed and increased from zero to right as shown by arrows. Curve *B:* potentials decreased from right to zero, while the current continued to flow. There is a hysteretic effect, the resistance remaining higher than during the increase of potential. To left of zero, however, with increase of potential during current flow, Curve *B* agrees well with Curve *A*. Curve C was taken after 0.5 M KCl had been substituted for sea water outside the membrane. The curve goes through a double inflection near the zero line. Potentials to left of zero cause *outward* current, to right, *inward.*

rising again at higher potentials. When 0.5 M KCl was substituted for sea water on the outside of the membrane the curve went through a double inflection at the lower potential values. The remainder of the curve paralleled the sea water curve at slightly different absolute values. This double inflection was characteristic of several membranes just after solutions had been changed and is undoubtedly due to the establishment of new diffusion gradients in the membrane.

Another anomaly, shown in Fig. 5, is the different shape of the curve on ascending and on descending potentials. In the descending arm (*B*) of the sea water curve, the current was allowed to flow continually while the potential was decreased, not being broken between readings. These were made in the standard time (15 seconds) after the new potential was established across the membranes. It is seen that the resistance stayed at a much higher value at most potentials, and then began to decrease more rapidly, at the approach to zero. On the reversal of potential the original values were nearly duplicated.

This delay, which resembles a hysteretic effect, is seen in another way when potentials are applied in rapid succession. While the same resistance level is eventually reached, this takes longer at the first application than at later ones following immediately after. Fig. 6 is a string galvanometer record of the bridge deflections, showing two applications of the same potential to a membrane separating solutions of different conductivity. In this case the less mobile ions were being carried into the membrane by the current and the resistance rose during the flow of current. But the time to rise approximately to balance point (at break) was nearly 14 seconds, while the same point was reached in about 7 seconds on a new application, following 1 second after the first was broken. It is evident that during the 1 second between potential applications much diffusion had occurred, which had reduced the resistance again, and unbalanced the bridge; but this had not gone as far as in the original condition, and it did not take as long to establish the new gradient in the membrane. Very similar hysteretic effects have been noted in *Valonia* cells,[7] and the curves have much resemblance.

Comparison of this record with those of living cells does, however, show a striking difference as respects the back E.M.F. of polarization. When present this appears on the string records as a large upward excursion of the image at the removal of an applied potential. In

Nitella[13] and in normal *Valonia* this upward curve at "break" is nearly symmetrical with the downward movement at "make:" in this it resembles the curves for electrode polarization. But the upward movement at break is nearly absent in the records for collodion, showing that the rise of resistance is one of "real" or ohmic resistance.

In this respect these collodion membranes are in agreement with those of Espermüller[14] which were held only between metal plates, without any contact with solutions. The ions were there necessarily derived from the collodion itself, and the resistance rose or fell as they were carried to one side or the other of the film. Polarization was absent.

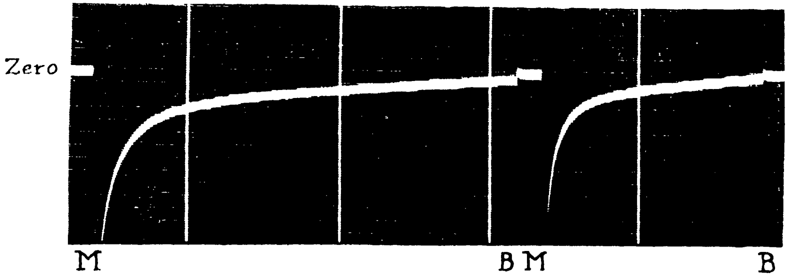

FIG. 6. String galvanometer record of bridge deflections during two applications of 0.5 volt to a membrane separating 1.0 M KCl from 1.0 M HCl. (Positive current from KCl to HCl.) The image deflects from zero (no current in bridge) downward at the application or "make" (*M*) of the potential and rises as the resistance increases nearly to the balance point. At removal of the potential or "break" (*B*) there is a negligible back E.M.F. displayed as the image returns to zero. At the second make (*M*) the deflection is less and the resistance rise is about twice as fast. At break (*B*), there is again a negligible back E.M.F. Time marks, 5 seconds.

IV

The biological significance of the phenomena described for collodion will depend upon the relative rôles played in living cells by the two components of any hindrance to the passage of direct current. These may be (1) a real ohmic resistance and (2) an E.M.F. of polarization.

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[13] Blinks, L. R., *J. Gen. Physiol.*, 1929–30, **13,** 495.

[14] Espermüller, E., *Arch. Elektrotech.*, 1928, **21,** 148.

Both may occur in any conducting system and certainly the first is always present (with a possibility of variation). It would be possible to ascribe many of the observed resistance variations in living matter indirectly to variations in the bioelectric potential difference existing at the cell surface, which, by a small change of value (as at or near the zero point in these curves), might bring about a large change in the electrolyte content of that surface. This would affect not only direct current measurements, but also those of low frequency alternating current in which the ohmic resistance of the membrane is more important than its capacity. A tempting hypothesis could thus be established on the relations between bioelectric potential and electrical resistance.

The evidence, however, for *Valonia* and *Nitella*, as well as for many other organisms, indicates the greater importance of polarization E.M.F.'s in the observed "resistance" to direct current. The study of impaled *Valonia* cells[11] especially indicates the important rôle of these back E.M.F.'s. It is felt therefore that the mechanism of rise of resistance in the thin collodion films reported here does not completely represent that of the cells. It is, however, described since it may have a bearing upon other cases. It should further be noted that to a certain extent collodion furnishes a model for polarization potentials, inasmuch as Labes and Zain[5] found back E.M.F.'s of as high as 80 mv. developed by long passage of current through another type of membrane. Further study of these as a model of the *Valonia* cells is in progress.

SUMMARY

The resistance of very thin collodion membranes to direct current bears some resemblance to that of living cells since it varies with the applied potential. With membranes separating two different solutions the resistance varies with the direction (and the voltage) of the applied potential, rising when less mobile ions are carried across the membrane and falling when more mobile ones are so carried. (With some membranes the resistance varies with potential when the same solution is on both sides.) These changes are very prompt and regular. There is a hysteretic effect of previous current flow.

But the membranes differ from *Valonia* cells in that the rise of resistance is largely ohmic, there being little or no polarization potential.

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THE VARIATION OF ELECTRICAL RESISTANCE WITH APPLIED POTENTIAL

III. IMPALED VALONIA VENTRICOSA

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I

In many freshly gathered cells of *Valonia ventricosa* the apparent resistance of the protoplasm to direct current varies with the value of the potential applied to measure it.[1] It was further shown[1] that the apparent resistance of a single layer of protoplasm in such cells depends also upon the direction of the current, falling somewhat when the applied potential is increased to carry the positive current outward across it, but rising greatly during the first increase of 100 mv. in the opposite (inward) sense.

These directional effects were studied in cells in which the contact-region at one end had been killed with chloroform. They were therefore limited to the time during which the intact end remained uninfluenced by the diffusion of chloroform, or by the death of the other end. This was at most 15 minutes to half an hour. In order to extend greatly the time of these measurements across a single layer of protoplasm, and to avoid the influence of toxic agents, a series of resistance measurements was made with *V. ventricosa* cells impaled upon fine glass capillaries. Through these the current was led into (or out of) the vacuole, and passed but once across the protoplasm to (or from) the surrounding sea water.

It is recognized that the advantages of this method are balanced by the possible injury inflicted by the insertion of a capillary into the cell. It has already been noted[2] that cells which have reached the

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[1] Blinks, L. R., *J. Gen. Physiol.*, 1929-30, **13**, 793.

[2] Blinks, L. R., *J. Gen. Physiol.*, 1929-30, **13**, 361.

"constant" state may be rendered again "variable" (*i.e.*, having an apparent resistance depending on the value of the applied potential), simply by impalement. However, the production of this state at will has contributed toward an understanding of its causes, and it is believed that an objective statement of the changes occurring as the cells remain impaled may be valuable. The formation of an "electrical seal" about the capillary has already been referred to in the static measurements of Osterhout, Damon, and Jacques.[3],[4] The present measurements of resistance and polarization during the flow of current across the whole protoplasmic film yield further criteria on the condition of impaled cells.

II

The present technique was directly adapted from that of Osterhout, Damon, and Jacques, the impalement being done under a slight pressure and a small hydrostatic head subsequently maintained to prevent the collapse of the cells. Various arrangements utilized are shown in Figs. 1, *a*, *b*, and *c*. Supported by the capillary above, and by a ring of cork or glass below, the cell was held gently and motion upon the capillary was prevented. This immobility contributed largely to the prompt recovery and long life of the cells. The use of rather small cells (from 0.5 to 1 cm in diameter) was also helpful, since the protoplasmic film of larger cells seems to be much more unstable, and breaks up into hundreds of tiny cells (inside the old cell wall) very promptly after mechanical injury such as pricking. Smaller cells (2 to 3 mm. in diameter) could sometimes be impaled without support from below (Fig. 1 *d*).

The capillaries usually projected 2 or 3 mm. into the vacuole; they were about 1 cm. long and had an outside diameter of 0.2 to 0.5 mm. They were drawn on the ends of ¼ inch (or smaller) glass tubing, which, filled with artificial sap of *Valonia ventricosa*,[5] formed the salt-bridge connecting the vacuole to one of the electrodes.

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[3] Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927-28, **11**, 193.

[4] Damon, E. B., *J. Gen. Physiol.*, 1929-30, **13**, 207.

[5] This had the composition: 0.6 M. KCl, 95 parts, to 0.6 M. NaCl, 5 parts. For the original analysis of this sap see Cooper, W. C., Jr., and Blinks, L. R., *Science*, 1928, **68**, 164.

In this they resemble most of the so-called "micro-electrodes," which are generally microscopic only as respects the capillary point inserted into the cell, and are more properly "micro-salt-bridges" connecting the cell interior to more or less distant electrodes of quite ordinary size.[6] This is indeed a more satisfactory arrangement than a minute metal surface, in any electrical measurement where polarization is to be avoided; in the present studies particularly, where current flow is purposely caused in order to produce the described effects, such polarization

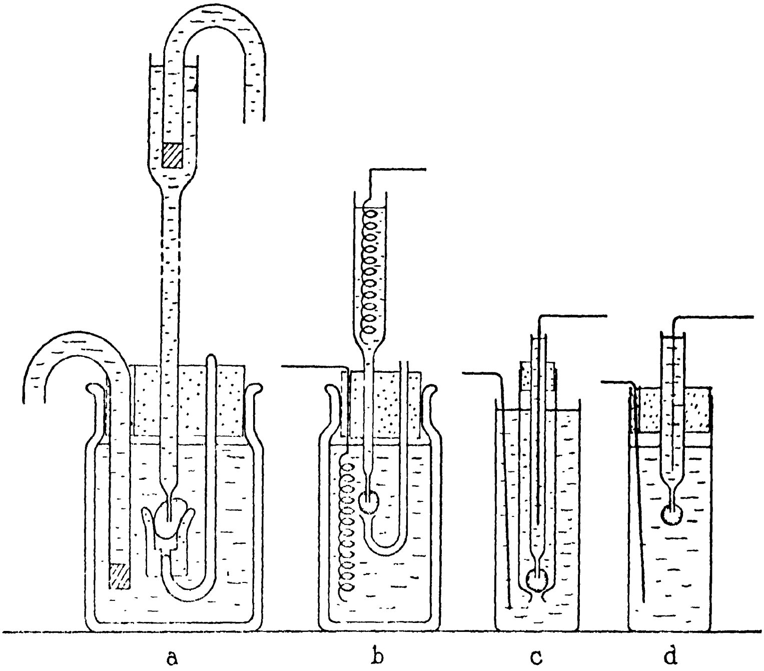

FIG. 1. Apparatus used for supporting the impaled cells of *Valonia*. In each case the capillary is drawn on the end of a vertical tube which fits rather tightly in the cork stopper. It is filled with artificial sap. In *a* the cell is supported from below on a cork with flexible prongs, itself supported on a glass rod inserted firmly into the cork stopper. In *b* the support is the flared end of a glass tube similarly inserted. In *c* the cell is supported inside the narrow orifice of a wider glass tube. In *d* there is no lower support for the small cell. Except in *a* the electrodes are of straight or coiled silver wire, inserted into the sea water or artificial sap as shown. In *a* there are agar-plugged salt bridges connecting to lead electrodes (not shown).

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[6] But the silver plated quartz needle recently described by Sen is really a micro-electrode. Sen, B., *Proc. Soc. Exp. Biol. and Med.*, 1929-30, **27**, 310.

is inadmissible. It was found that very small electrodes of fine silver wire, chloride-coated and introduced through the capillary into the vacuole of impaled cells, gave very erratic results and seriously distorted the observed effects. There was also the danger of injury to the protoplasm by minute amounts of silver or other salts dissolving from metal electrodes inserted directly into the vacuole for long periods of time, and the impossibility of cleaning and recoating the surface at frequent intervals while in the cell. Instead, more distant electrodes of much larger surface were employed and the desired currents obtained through the capillaries by the application of higher voltages.

The electrodes were usually of straight or coiled silver wire (20-gauge) several inches in length, and inserted respectively into the sea water and into the artificial sap in the tube above the capillary (Figs. 1 *b*, *c*, *d*). They were cleaned and recoated with chloride electrolytically at least once a day (or oftener when their polarization, measured with a blank capillary, warranted). In some of the studies even larger electrodes of sheet lead, amalgamated and chloride-coated, were employed; these dipped into separate vessels and were connected to the cell by agar-plugged salt-bridges, shown in Fig. 1 *a*.

At either type of electrode, polarization was virtually absent during long flows of the largest currents used in the measurements (50 to 100 microamperes). Similar constancy of ohmic resistance was observed during such flows of current through the capillaries, either when these were in connection with artificial sap in a vessel, or with the natural sap of the vacuole. Thus in a cell killed immediately before measurement, the current was passed through a capillary inserted into the vacuole, for as long as 15 minutes, and in either direction, without significant change of resistance. Such constancy is of course essential for determining the amount of change in the living protoplasm. Very satisfactory agreement of this basal value from day to day was likewise found, the only fluctuations being such as were expected from 2° or 3° changes of temperature.

The constancy of this resistance disappeared when the sap-filled capillaries were dipped directly into sea water, or remained in a dead cell long enough for the sea water to diffuse into the vacuole. In these cases the resistance rose when the positive current was passed inward, and reached a value about 20 per cent higher when most of the K^+ ions had been supplanted in the capillary by the less mobile ions of the sea water. Such a diffusion of sea water into the sap is a sign of gross injury, and this effect was never found in healthy cells. Since the rise of resistance in the capillary is entirely ohmic, it is easily distinguished (by the absence of back E.M.F.) from the protoplasmic phenomena, which will later be shown to be largely reactive (i.e., due to the production of a back E.M.F.).

Further check on the basal resistance of the capillary was given by alternating current measurements at a frequency (5000 cycles) where the impedance of the protoplasm was negligible. These gave very constant readings from day to day, independent of the changes of apparent resistance to direct current occurring in the protoplasm.

Depending upon the diameter and length of the capillary, this basal resistance (*S*) ranged from 10,000 to 100,000 ohms. In order to pass sufficient current through such capillaries to obtain the described effects in the protoplasm it was sometimes necessary to apply as much as 4 or 5 volts to the electrodes. The potential drop across the protoplasm was, of course, but a small fraction of this. The total applied potential (*V*), and the apparent direct current resistance (*R*), were determined in the Wheatstone bridge previously described.[2] This bridge also provided for the measurement of potential differences existing in the cell, either by compensating to zero with a series potentiometer, or by deflection of the direct reading detector (vacuum-tube electrometer with string galvanometer). This could be done at full sensitivity, with one arm of the bridge thrown out (open circuit), or, in the condition of balance, *with sensitivity reduced one-half.*

The values of net protoplasmic potential difference were obtained by subtracting or balancing out the P.D. between the electrodes, due to the different chloride content of sea water and artificial sap. This P.D. was usually about 4 mv.

For purposes of comparison with previous papers,[1,2] the results with *Valonia ventricosa* are here described. The cells were used in June, July, and August at Dry Tortugas, Florida, at temperatures between 25° and 30°C., and later in New York at lower temperatures with good agreement of the results.

Further general agreement has been found in the behavior of impaled cells of *V. macrophysa* in the course of continued study at Bermuda, except that these cells show even larger polarization potentials, and recover more readily to a condition resembling the "constant" state of intact cells. These are reserved for discussion in a later paper.

As before, it is a pleasure to acknowledge the generous co-operation of the Carnegie Institution of Washington in offering the facilities of its laboratory at Tortugas.

III

This section describes the characteristic values of potential difference naturally existing across the protoplasm of *Valonia ventricosa.* As indicated in a preliminary report,[7] these differ somewhat from those of *V. macrophysa* as described by Osterhout, Damon, and Jacques.[3,4] Theoretically the values of bioelectric potential, developed by the protoplasm in contact with different solutions, should have a close relation to the polarization potentials elicited by the flow of current to or from such solutions. This expectation has been justified in the correlation found between several types of polarization responses and the bioelectric potential which accompanies them.

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[7] Blinks, L. R., *Carnegie Inst. Washington Year Book*, 1928–29, 28, 277.

We may indicate five typical stages of the bioelectric potential difference:

(1)

Initial mechanical shock—P.D. 5 mv. or less (inside positive).

This stage follows immediately after impalement and lasts from a few minutes to several hours, depending apparently on the degree of injury sustained. The earliest measurable P.D. (some 10 seconds after impalement) is about 5 mv.; this usually drops within a few minutes to nearly zero. There is then a slow recovery of 1 or 2 mv. per hour, this process passing into stage (2) without apparent break. But in occasional cases there may be a complete reversal of P.D. to about 30 mv. (*outside positive*). This usually occurs promptly after impalement, lasts but a few minutes and is as promptly recovered from. It seems to occur most frequently in cells which have already reached the "constant" state before impalement, and it is really a momentary example of stage (4), probably elicited by the breakdown of the outer layer (X) of the protoplasm.[4] As X recovers or Y in turn breaks down, the P.D. returns to zero and the remaining slow recovery process ensues.

It may be noted that the potential differences given for stage (1) are in good agreement with those observed by Taylor and Whitaker[8] soon after the insertion of a micro-electrode into the vacuole of *Valonia ventricosa*. They state that there was "a relatively rapid lowering of the potential difference, which was then followed by a gradual rise to a maximum . . . . Although the amount of voltage varied for different cells, we have estimated a mean value of the potential difference between the cell sap of *Valonia* and sea water to be about 0.002 volt. The charge of the cell sap of *Valonia* was plus with reference to sea water." Had they followed the process longer there can be no doubt the P.D. would have been found to rise to values typical of stage (2). The necessity of such long waits for impaled cells to become stabilized was insufficiently realized for a long time, and probably also accounts for the low values (1 or 2 mv.) reported by Osterhout,[9] who first measured the P.D. in this manner in *Valonia*, and by Jost[10] who repeated them. Umrath[11] has recently stated that his earlier reports for *Nitella* were likewise too low for the same reason. This need of recovery was first pointed out by Osterhout, Damon, and Jacques.[3] In early experiments of the author to measure the electrical resistance of impaled cells, great ambiguity was met through similar neglect of the time factor, and the method was temporarily abandoned.

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[8] Taylor, C. V., and Whitaker, D. M., *Carnegie Inst. Washington Year Book*, 1925–26, **25**, 248.

[9] Osterhout, W. J. V., *J. Gen. Physiol.*, 1925–26, **7**, 561.

[10] Jost, L., *Sitzber. Heid. Ak. Wiss.*, 1927, Abh. 13, 1.

[11] Umrath, K., *Protoplasma*, 1930, **9**, 576.

(2)

The stage of recovery and normal potential: P.D. from 8 to 25 mv. with an average of 15 mv. (inside positive).

This is reached within several hours to 2 days after impalement, and persists through the life of the cell, with a gradual decrease of value.

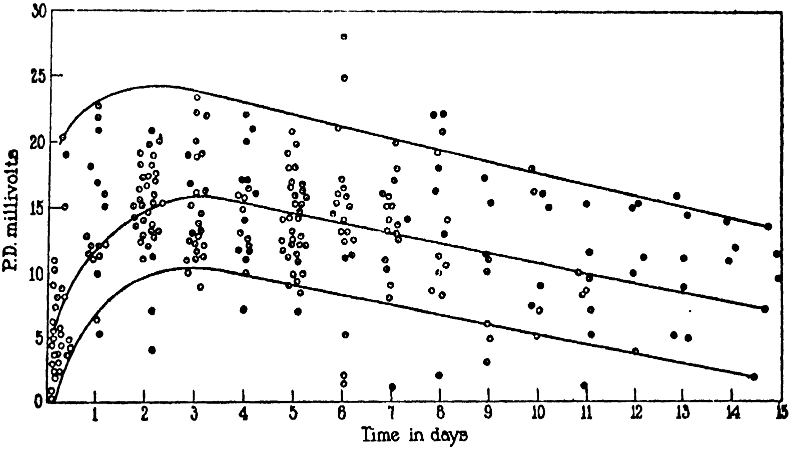

FIG. 2. Composite graph of the P.D. measured across the protoplasm during the life of 34 impaled cells of *Valonia ventricosa* in sea water. Each point is a single determination. The outer lines roughly enclose nearly all the scattered values, and the median line is drawn freehand to represent an average value. The P.D. is that of the vacuole, which is positive in the electrometer circuit to the sea water.

Fig. 2 gives a composite picture of the course of these values for a representative group of cells. The variations from day to day might be several millivolts, but in general a given cell had a more or less constant P.D., and those which were apparently most healthy (*i.e.*, dark green, turgid, and shiny) gave the highest values.

It should be noted that this P.D. is in the same direction as that of *V. macrophysa* similarly impaled and immersed in sea water, but has a normal value from 2 to 3 times as great. This is a specific difference, which if connected only with the higher potassium content of the sap of *V. ventricosa*, must argue *against* a high mobility of K$^+$ ion in the protoplasm, at least at the inner surface, since the P.D. [illegible] increased in the wrong direction, the positive current tendi[illegible]w from sea water *inward* to the sap. The difference can p[illegible] explained on the basis of 3 layers in the protoplasm.[4]

While stages (1) and (2) are the only ones necessarily passed through by the living impaled cells, there are two other stages which may temporarily be produced by special causes, and have important relations to the polarization phenomena. These are:

(3)

A stage of increased P.D. (inside positive), produced by the application of various solutions. The highest of these measured was:

a) 100 millivolts (inside positive), produced by the immersion of the cell in artificial sap (such as employed in the capillary tube[5]). This is almost instantly attained, remains from 1 to 5 minutes, and then more slowly falls away to 5 mv. or less (inside positive). Sometimes the initial rise is only to 40 or 50 mv. There has not yet been found the secondary rise shown by *V. macrophysa* at its first contact with its own sap, and this behavior more resembles the succeeding applications of sap to that species.[4] The effect may be produced time after time in *V. ventricosa* if the cell is returned to sea water for several hours between exposures to sap. The rise of P.D. might be attributed to the high mobility of the K$^+$ ion in the *outer* surface of the protoplasm, and its succeeding fall to the total imbibition of the latter with KCl destroying the gradient of K$^+$ concentration across the outer surface. Protoplasmic asymmetry might thus be said to exist after the exposure of cells to sea water, but to disappear upon their imbibition with sap. According to Damon's explanation[4] for the shape of curves in successive applications of sap to *V. macrophysa*, the middle layer (*W*) of the protoplasm in *V. ventricosa* might further be considered always fairly well imbibed with KCl. 0.6 M KCl acts much like sap in these experiments.

b) In addition to the large temporary increase of P.D. produced by sap, other, smaller, increases were produced by immersion of the cells in diluted sea water (1/10 sea water to 9/10 isotonic glycerine), and in 0.6 M $MgSO_4$. In each case the P.D. rose to 35 or 40 mv. (inside positive) and remained up for half an hour or longer. This increase is very much like the effect of dilute sea water on *V. macrophysa*.[12] The direction of this increase argues for a higher mobility of some anion, presumably Cl^-, in the inner surface of the protoplasm, since the gradient is greatly increased by these treatments. Immersion of the cells in 0.6 M NaCl promptly brings the P.D. nearly to zero, where it remains, and this could be ascribed to the abolition of the Cl^- gradient.

We now come to a fourth typical and very interesting stage:

(4)

Reversed potential: P.D. to 50 mv. (*outside* positive).

This frequently occurs after a long exposure to dilute sea water or $MgSO_4$ solution, especially just after the cells are re-immersed in ordinary sea water. The change to this level is very rapid, sometimes being completed in a few seconds. The recovery from it is usually somewhat slower, and its total duration is seldom more than 5 or 10 minutes. Its occurrence shortly after impalement in some cells has already been noted under stage (1). It may also be elicited after very large current flows, *either* of inward or of outward current. For the latter it is not in the direction of a counter E.M.F. and therefore is distinct from the polarization response. Its independence of the current direction, and its production under a variety of circumstances, suggests rather that it is in the nature of an "alteration" such as the temporary destruction of the outer protoplasmic layer (*X*)[4] (or the reduction of its E.M.F.), leaving the outwardly directed E.M.F. of *Y*. This alteration is not permanently injurious to the cell, and is indeed characteristically found in those cells most nearly "normal" or "constant" in their apparent resistance. Thus it is most frequently seen just after "constant" cells are impaled, and then again toward the end of stage (2) when the cells have recovered from the injury of impalement. The ease of its production may then actually become troublesome in the measurement of bioelectric potentials.

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[12] Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 445.

(5)

The abolition of potential: (P.D., 1 mv. or less).

This stage is reached on the death of the cell even before the protoplasm is obviously disintegrated. It may be produced by long applications of single salt solutions, or by heat, or by chloroform and other toxic agents. It is of no particular biological interest therefore, but serves as a check on the electrodes and on the experimental technique.

IV

We may now turn to the phenomena caused by the flow of current at these different stages.

Stage (5) has already been referred to in the discussion of methods, and serves as a convenient check upon the constancy of the electrodes and of the capillary resistance. In impaled cells just killed by chloroform, as much as 5 volts may be applied to the electrodes, and an approximately constant resistance measured during as long as a half hour of current flow in either direction. Neither electrodes, capillary, dead protoplasm nor cell wall introduce any significant change of resistance during this time. This shows that the heating effect of the current is negligible, and that migration of sea water into the vacuole is small.

In stage (1) are found rather similar conditions, except that the resistances tend to be slightly higher than those of the blank capillary before its insertion, and there is a feeble reaction, shown in the detector as a small back E.M.F. at the break of the current (of either direction). Occasionally there is a slightly higher apparent resistance to a small outward current than to an inward current, especially in cells where the reversion to stage (4) occurs. In general the effective resistance of the freshly impaled cells is so low as to be negligible at all current densities. This unexpected result in the early experiments of the author led to the temporary abandonment of the capillary method, since it could not be reconciled with the high effective resistance of the same cell measured just before its impalement. The low resistance is almost certainly due to leaks around the insertion of the capillary, but also to more profound changes in the entire protoplasmic surface. The nature of these changes begins to appear in the recovery process.

Stage (2). If now, after the cell had been impaled several hours, or as much as a day, a small potential (0.25 to 0.5 volt) was applied to the electrodes, the behavior was even more like that of stage (5) or a dead cell. The small reaction (back E.M.F.) shown in stage (1) had usually disappeared, and the resistance was essentially equal to that of the blank capillary before its insertion (or to the alternating current resistance measured at the moment). When the potential was increased in a direction to carry the positive current *outward* (from sap to sea water) across the protoplasm, this low resistance was maintained, even up to currents of 100 microamperes (caused by the application of 5 volts to a capillary of 50,000 ohms resistance).

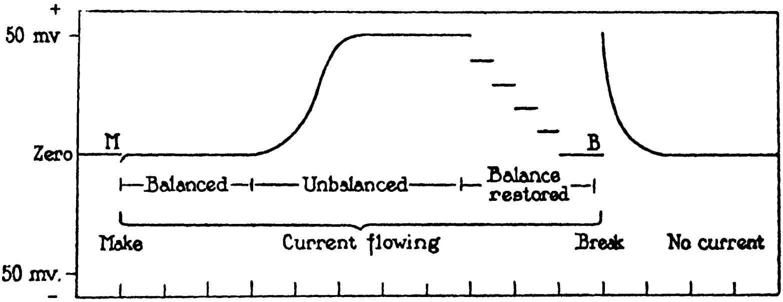


FIG. 3. Tracing from string galvanometer record showing deflections of the bridge detector during the flow of current inward across the protoplasm of an impaled cell of *Valonia ventricosa.* The upward movement is due to a rise of resistance, or to the appearance of an E.M.F. opposed to the applied potential. After the rise has occurred R is increased in 5 steps of 1000 ohms each to bring the detector to zero. At break there is a momentary upward movement due to the back E.M.F. $R = 45{,}000$ ohms, later increased to 50,000 ohms. Applied potential, 1 volt. Detector sensitivity in open circuit as shown. Time marks, 1 second. *M*, make; *B*, break. (P.D. of cell was compensated to zero before the record.)

Only when the current was reversed and passed *inward* across the protoplasm from sea water to sap was there a change of the apparent resistance. At slightly higher voltages, between 0.5 and 2 or more volts in these early stages, this change occurred rather suddenly (Fig. 3). At the critical voltage there was at first no response to the flow of current, but in from 1 to 20 seconds there began a rise of apparent resistance, at first slow, then very fast, then slower again, sometimes carrying the image of the galvanometer fiber out of the field. When this was brought back to the center by balancing the decade resistance box of the adjacent bridge arm, a steady state was now maintained as long as the potential was applied to the bridge. The amount of the rise varied from 1 or 2 per cent up to 10 per cent or more of the capillary resistance, and sometimes as much as 10,000 ohms additional resistance was required in the decade box to balance this apparent rise. What now occurs at the break of current, when the potential is removed from the bridge input terminals? In an ordinary resistance balance, through a wire resistance or through a blank capillary, the galvanometer image being brought to zero remains there

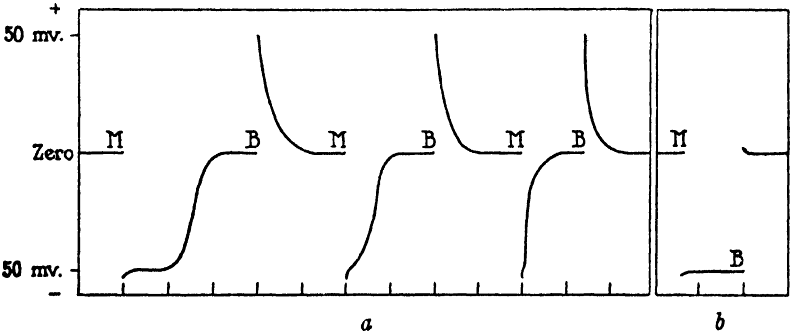


FIG. 4. Tracing from string galvanometer record showing deflections of the bridge detector during applications of 1 volt to the cell of Fig. 3. R = 50,000 ohms throughout. *a*, inward current, 3 applications. *b*, outward current. Detector sensitivity in open circuit as shown. Time marks, 1 second. *M*, make; *B*, break. (P.D. of cell was compensated to zero before the record.)

when the potential is removed from the bridge. But in this case the image deflected greatly at the instant the outside potential was removed, and then returned slowly to zero again. Herein the cell resembled a platinum electrode, or a large condenser, and the deflection at break was due to the accumulated charge, or polarization potential. This is shown in Fig. 3, together with the preceding rise of apparent resistance and the balance to zero.

If, now, the decade box increment being retained and the bridge balance kept at the steady state value reached in Fig. 3, we apply the same critical voltage again, the record of Fig. 4 ensues. A rather long wait has followed the "break" of Fig. 3, so that the string image is found now to be deflected approximately as far downward as it previously had risen upward. There is a delay of about a second at this level, and then the rise begins again, at first slowly, then more rapidly, finally flattening out at the center (zero) line representing the bridge balance. At removal of the potential from the bridge ("B") there is again an upward deflection, due to the back E.M.F. When this has returned fully to zero for about a second, the potential is again applied to the bridge and again there is a deflection downward, which is much more rapidly recovered from, but still shows the double inflection of the first curve. Again there is an upward deflection at "break," returning smoothly to zero. A third application after another second's delay gives a smooth curve which has almost no hint of a double inflection and is nearly an image of the return curve at break. It now closely resembles the charge and discharge curves of a condenser connected in the bridge, or the polarization curves of a blank platinum electrode. It is evident that an effect of previous flow of current persists from one exposure to the next, and that there has been a progressive change in the response of the protoplasm suggesting that of a change from a reversible electrode to a polarizing

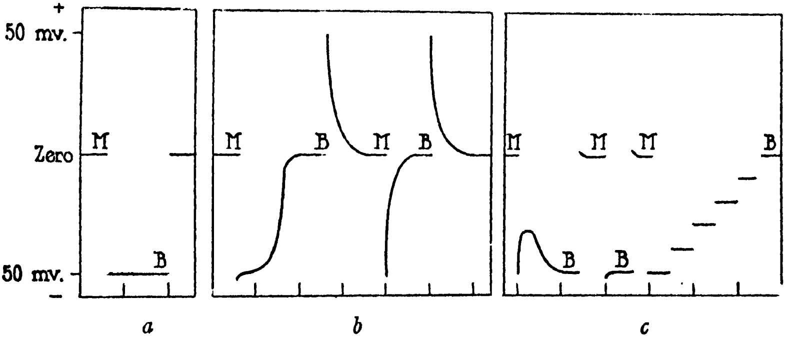


FIG. 5. Tracing from string galvanometer record showing deflections of bridge detector during applications of 1 volt to cell of Figs. 3 and 4, at a partially recovered state. *a*, Outward current; *b*, inward current; *c*, outward current. In the latter the first exposure causes a partial polarization, followed by a regression. *R* = 50,000 ohms until the 3rd exposure in *c*, when it is reduced in 1000 ohm steps to 45,000 ohms. Detector sensitivity in open circuit as shown. Time marks, 1 second. *M*, make; *B*, break.

electrode. This however, holds only for *inward* currents, and if the current be reversed through the cell we obtain the record of Fig. 4*b*. The galvanometer image deflects immediately downward, and stays there as long as the current flows. The downward deflection is due to the maintenance of the bridge balance at the augmented value of *R* caused by the inward flow of current. If the balance be returned to that of the basal resistance *S*, the string image returns to zero, and there are no appreciable polarization phenomena either at make or break (*i.e.*, the record is essentially a straight line). The protoplasm is apparently a perfectly reversible electrode to an outward current.

This condition may prevail for many days after impalement. But sooner or later the protoplasm begins to polarize also to an outward current, and this is foreshadowed by the behavior shown in Fig. 5. The first section (*a*) repeats Fig. 4*b* for the bridge balance to the augmented state; there is seen to be no appreciable polarization with an outward current. The direction of the current through the cell was now reversed and the prompt polarization characteristic of the inward current was produced (Fig. 5 *b*). The exposure was repeated, with even quicker response. Now the current was again quickly reversed, and Fig. 5 *c* shows the response occurring with an outward current, followed by an immediate regression to the level of Fig. 5 *a*. On break there is little or no back E.M.F., the image returning directly to zero without an excursion above the line. On the second application there is not even a temporary polarization, and the deflection is instant and complete. Finally the record shows the restoration of the bridge balance to the basal value (*S*) in steps of 1000 ohms.

It is evident from this record that the protoplasm is still not ordinarily polarizable to an outward current, but may be made so temporarily. Either by the movement of ions out of the protoplasm into the vacuole, or by the alteration of the protoplasmic properties, the previous inward current has left an effect which persists a short time. The further recovery process in the course of stage (2) has the same effect as this flow of current and Fig. 6 shows the response given at a later time by a cell that is beginning to polarize normally with currents in either direction. Fig. 6 *a* shows the prompt and symmetrical charge and discharge curves with the inward current. Fig. 6 *b* shows nearly an equal response with an outward current, but there is a succeeding regression which begins at a lower potential than with the inward current. This in general represents the behavior of the cells for the remainder of their life upon the capillary. They give good polarization with as low a potential as can be applied, and, for values up to about 0.5 volt (depending upon the resistance of the capillary) have a nearly constant apparent resistance with the current either direction. Above this value the apparent resistance may fall off, more rapidly with outward currents than with inward ones. The breakdown process is much the same in either case, being a regression of polarization following a preliminary rise, and simply occurs at lower current densities in the outward direction than in the inward. In this the cells in the recovered condition of stage (2) resemble "constant" intact cells which have been chloroformed at one end, and are considered to have reached as nearly normal a state as the impaled cells ever do, having an apparent resistance that is independent of the applied potential up to the breakdown value.

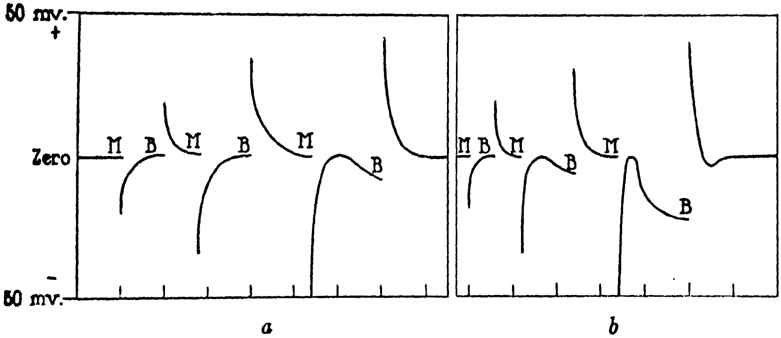
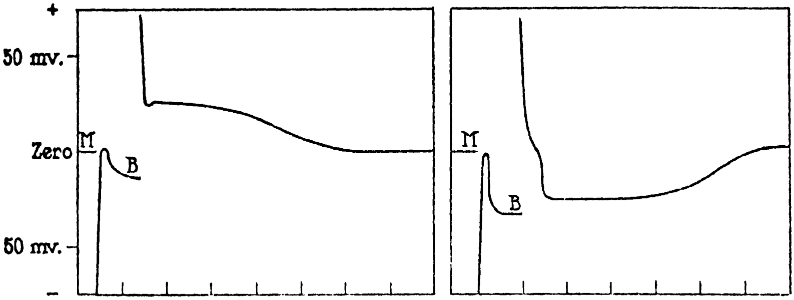


FIG. 6. Tracing from string galvanometer record showing deflections of bridge detector during applications of increasing voltages to an impaled cell of *V. ventricosa* which has reached the "constant" state. Applied potentials: 0.5, 1.0, and 1.5 volts, in the order given. *a*, Inward current; *b*, outward current. Detector sensitivity as shown (open circuit). Time marks, 1 second. *M*, make; *B*, break.

In stage (3), the condition of increased P.D. across the protoplasm, the relations of stage (2) still hold quite well. Most remarkable of all, the polarization response obtained when the cell is immersed in artificial sap is about as great as when in sea water, and is characteristically even more rapid in its development, having a very abrupt rise. After the exposure to sap has been continued for some time the polarization becomes less, and eventually may even be larger with an outward current than with an inward one. But for as long as half an hour after some cells have been immersed in artificial sap, their polarization response remained large and their apparent resistance as high as in sea water. It is difficult again to reconcile this result with a high mobility of K^+ ion across the entire protoplasm, and it may become necessary to ascribe the effects to some unspecified ion, possibly existing only within the protoplasm, and not dependent upon the environment immediately supplied in the experiment.



a *b*

FIG. 7. Tracing from string galvanometer record showing deflections of bridge detector during applications of 3 volts to the cell of Fig. 6. *a*, Inward current; *b*, outward current (in *b* the cell is reversed in bridge). In each case the appearance of the polarization E.M.F. at break is succeeded by stage (4) with a reversed P.D. of about 50 mv. (outside positive). $R = 38{,}000$ ohms; open circuit sensitivity of detector as shown. Time marks, 1 second. *M*, make; *B*, break.

Finally we come to the anomalies of stage (4). These consist both in its production, and in the polarization phenomena which occur when it is established. Its production mechanically (by impalement) and chemically (by dilute sea water, etc.,) has been described above. Fig. 7 shows its production after the passage of large currents; in *a* after an inward, in *b* after an outward current. After the inward current, it has the same direction as the polarization potential and may normally add to the value of this. Certainly some frequent distortions of the return curve may be ascribed to a momentary production of this stage. But in the present case it forms a very distinct level equal to about 50 mv., which persists for several seconds before returning to zero. In Fig. 7 *b*, after a large outward current has passed, there is first a large upward excursion due to the normal back E.M.F., but as this returns to zero there is a further movement, and the image goes below the zero line, again to an amount representing about 50 mv. This is now contrary to the polarization E.M.F., and is therefore in the same direction as the applied potential, which had driven the current *outward* across the protoplasm. The P.D. of stage (4) is thus in reverse of the normal potential and is *outside positive* to the electrometer. (In Fig. 7 *b* this P.D. appears in an opposite direction from Fig. 7 *a* because the cell has been reversed in the bridge.) Whether it is caused by the outward flow itself, or by the return current from the large polarization charge, is still uncertain. In either case it could be explained by the temporary alteration of the outer layer (X).[4]

While in these records the reversed P.D. of stage (4) lasted only a few seconds, in other cases it remained long enough for measurements of effective resistance to be made across the protoplasm while in this stage. As indicated before under stage (1), the usual polarization phenomena are now reversed, and the resistance appears to be greater to an outward current than to an inward one. This continues as long as the reversed P.D. remains, and then, as it begins to return to normal, polarization becomes more nearly equal in both directions and finally reaches that of the late stages of (2).

Study of the records has shown that the P.D. of stage (4) is frequently driven approximately to zero by an outward current. Since this upward movement is characteristic of a back E.M.F., it would therefore appear to be a polarization, and would be measured as an increased resistance. On the other hand, the P.D. of stage (4) is but little increased by an *inward* current, and there is almost no back E.M.F. developed in excess of the existing P.D. (which, of course, is taken as the null point, either balanced out or used as a pseudo zero). Thus an apparent polarization of as much as 40 or 50 mv. to an outward current may really be due to the destruction of so much P.D. This illustrates a pitfall inherent in these measurements, which may have to be taken account of in other biological "polarizations." That it is not entirely responsible for the total polarization to an outward current has, however, been satisfactorily shown in *Valonia macrophysa*, and will be considered more fully in a paper dealing with that species. Since an increased polarization to an outward current sometimes appears during the exposure of cells to artificial sap, when there is no reversal of P.D. across the protoplasm, further interest is added to its study.

One question may remain concerning the recovery of impaled cells to a "constant" state. May not the polarization apparently occurring with currents directed outward really be due to their passage inward across a film of protoplasm formed over the orifice of the inserted capillary? Aside from the difference of constant intact cells which have been chloroformed at one end, and which show much the behavior of recovered impaled cells, there is the great difference of current densities between the point of the capillary where the current enters the vacuole and over the general surface by which it leaves. The area of protoplasm formed over the end of the capillary could not be more than 0.1 mm.^{2} while that of the cell surface is usually from 2 to 3 cm.^{2}—a more than thousandfold difference. It is practically assured therefore that the relatively immense current density—a thousandfold greater—across the smaller protoplasmic film, if any, would break this down and render its resistance and reactance negligible. A ten to hundredfold increase is sufficient to do this on the remaining surface, as will be shown in a later paper dealing with injury and recovery. There is also the fact that such a protoplasmic plug would be bathed on its outer surface with artificial sap from the capillary, which is quite sufficient to destroy the protoplasmic properties of normal cells after exposures of a day or two.[9]

Still better evidence that such a film is not concerned is fortunately available, due to the straight open tubes employed in connection with the capillaries. With cells which had recovered to the "constant" state a fine platinum wire (attached on the end of a glass rod) was carefully inserted from above into the capillary, and a short distance into the vacuole. This was moved up and down gently and must have broken any plug which had formed. When the measurements were now repeated immediately after this probing the polarization effect was the same as before, and perfectly representative of the "constant" state as defined.

V

It has already become sufficiently evident that the apparent rise of resistance in the impaled cells is not real or ohmic, but is due to a polarization potential. This, decreasing the current through the system by opposing the applied potential, is therefore measured as increased resistance. The reality of this back E.M.F. is evident from the string galvanometer records, and its responsibility for practically all of the apparent resistance rise is shown by the essential constancy of the alternating current measurements throughout the life of the impaled cells, independent of their effective resistance to direct current.

This being so, the value of the polarization potential (in millivolts) becomes of more interest than the apparent resistance rise as measured in ohms. Several ways of determining this--by direct reading in a calibrated electrometer, by compensation with an opposed series potentiometer, or by measurement of the galvanometer deflection at the instant of break, as recorded photographically—have been employed, and will be reported upon in the paper dealing with *V. macrophysa.* These methods all agree well with the calculation of the back E.M.F., (P), from the known values of the applied potential (V), basal resistance (S), and apparent resistance (R), in which form the data for *V. ventricosa* were mostly recorded.

Thus in Fig. 3 where

V = 1.0 volt
S = 45,000 ohms and
R = 45,000 ohms (at the initial balance),

there is at first no back E.M.F. and hence no deflection of the detector. As the back E.M.F. builds up, the detector deflects upward, and reaches an apparent value of approximately 50 mv. Since the sensitivity of the detector to a P.D. in the cell is reduced one-half with the bridge closed,[2] this P.D. is therefore about 100 mv. The polarization potential appearing at "break" is also seen to go momentarily to this value.

When now the bridge was balanced to the augmented steady state, in the five steps of 1000 ohms each, shown in Fig. 3.,

V = 1.0 volt
S = 45,000 ohms, but
R = 50,000 ohms

By the equation

$$P = \frac{R-S}{R} \cdot V$$

the polarization potential $P = \frac{5000}{50{,}000} \cdot 1.0 = 0.1$ volt or 100 mv., which agrees with the directly-observed potential.

TABLE I

Value of Polarization Potential in the Protoplasm of Impaled Cells of Valonia ventricosa. (Calculated from Apparent Resistance to Direct Current)

Stage	Description	P.D. of cell before current flow		*V* Applied potential causing current:		*S* Basal resistance of capillary, etc.	*R* Apparent direct current resistance of capillary plus cell	*P* Back E.M.F. of protoplasm
		In +	Out +	Inward	Outward			
		mv.	*mv.*	*volts*	*volts*	*ohms*	*ohms*	*mv.*
1	Immediately after impalement	3.0		0.5		46,600	46,600	0
				1.0			47,000	8.5
				2.0			46,900	8.5
					0.5		46,700	1.0
					1.0		46,900	6.4
					2.0		47,000	17.0
2	Shortly after impalement	20.0		0.5-1.5		26,200	26,200	0
				2.0			27,500	95
				2.5			27,200	92
				3.7			26,900	94
					0.5-3.7		26,200	0
	Partially recovered	15.0		0.5		22,500	25,000	50
				1.0			25,300	110
				1.5			25,100	155
					0.5		23,000	11*
					1.0-1.5		22,500	0
	"Constant" state	12.0		0.25		35,500	38,000	16.5
				0.5			38,000	33.0
				1.0			38,000	66.0
				1.5			37,500	80.0
					0.25		38,000	16.5
					0.5		38,000	33.0
					1.0		37,500	53.0
					1.5		37,000	61.0
3	In artificial sap 5 minutes				1.5	26,200	27,200	55.0
		5.0		1.5			27,300	60.0
	In artificial sap 20 minutes	1.0		1.5-3.7	1.5-3.7		26,200	0
4	P.D. reversed by dilute sea water			0.25		56,000	56,200	1.0
				0.5			56,100	1.0
			45.0	1.0			56,000	0
					0.25		65,000	34.5
					0.5		61,000	41.0
					1.0		59,000	51.0
5	Cell killed by chloroform		1.0	0.5-3.0	0.5-3.0	45,400	45,400	0

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* Polarization occurs at first application only, and is followed by a regression to zero.

Table I gives further values of polarization potentials calculated by this means. It is seen that in the early stages of impalement these values are very small for small applied potentials, but increase suddenly and may reach values of 150 mv. or higher with inward currents. Later, smaller polarization potentials appear with currents of either direction, and for part of the range, are closely proportional to the values of the applied potential. This has the effect of producing a uniform apparent resistance and the cells may be called "constant."

We may now with profit go back to the results with intact cells of *Valonia*,[1,2] and consider their apparent direct current resistance in the light of polarization potentials caused by the flow of current. Always taking account of the cell wall forming a constant shunt around the protoplasm, the apparent resistance of the latter was found to vary from less than 1000 ohms, in the freshly gathered "variable" cells,[1] to over 50,000 ohms in cells which had attained constancy of resistance.[2] The dead resistance on the other hand was usually 50 ohms or less. Inserting this value (50 ohms) as S, the real ohmic resistance in the preceding equation, we find in every case a very large factor to be multiplied into the applied potential (V) to give the back E.M.F. (P). Thus when $R = 1000$ ohms and the applied potential is 20 mv., the back E.M.F., $P = \frac{950}{1000}\cdot 20$, or 19 mv. When, under the application of 100 mv., the resistance of the protoplasm in the variable cells has risen to 10,000 ohms,[1] an even larger factor results, and the back E.M.F., $P = \frac{9{,}950}{10{,}000}\cdot 100 = 99.5$ mv. In "constant" cells having an effective protoplasmic resistance of 50,000 ohms under the application of all potential differences from 10 to 100 mv., the back E.M.F.'s therefore so closely approach the applied potentials as to be essentially equal. The fact that these resistances begin to fall off at values between 100 and 150 mv. shows that in the intact cells the maximum polarization potentials are about half these values (since two layers of protoplasm are measured in series). This agrees well with the maximum values of 50 to 75 mv. developed by either inward or outward current in impaled cells which have reached a "constant" state. On the similarity of these potentials should the comparison between intact and impaled cells be based, rather than upon the apparent resistance levels. For it is obvious that while a back E.M.F. of 49.9

mv. can effectively reduce to nearly zero a current through the protoplasm when 50 mv. is applied to an intact cell, such a back E.M.F. developed by the same initial current density in an impaled cell can only partly counteract a potential of one-half volt applied through the capillary. Thus a much larger current will continue to flow in the latter case than in the former, and to these differences of continued current density may be attributed some of the differences found between intact cells and impaled cells. The study of *V. macrophysa* has been particularly directed toward these differences, and the polarization potential will there be further considered in relation to the current density across the protoplasm.

The *quantity* involved in these effects, as distinct from the *intensity* (E.M.F.) of polarization emphasized in this paper, is the *capacitance* of the protoplasm in its function as a condenser acquiring a charge during a flow of current. It early became evident that the capacitance of *Valonia* cells is very large, *i.e.*, the *quantity* of electricity taken to charge them to a given potential is large and hence a long flow of current is required. Otherwise the records of charge and discharge could not be taken with a (relatively) slow-period instrument such as a string galvanometer. The fact that this instrument gives good records of these curves at quite ordinary camera speeds (2 cm. per second) has greatly simplified their study. The time relations will be considered in a forthcoming paper along with the closely related alternating current measurements. But an indication of the quantities involved is given in such curves as are shown in Figs. 3, 4, 5, and 6. The time course of these is often a second or more (as compared to one or two *thousandths* of a second in human skin[18]) and the time constant τ (time for the potential of any part of the curve to change by $\frac{1}{e}$ of its value) may be 0.2 or 0.3 second. Since (when τ is in seconds, r in ohms, and c in farads) $\tau = r\,c$; and r, the total bridge resistance through which the potential is discharged, is usually not over 100,000 ohms, c, the effective capacitance of the protoplasm, is of the order of 2 or 3 microfarads. Thus for a cell of about 1 cm. diameter and some 3 cm.^{2} surface, this is as high as 1 microfarad per cm.^{2}

It is interesting to note that capacitances of this general magnitude were also found at the lower frequencies of alternating current, in experiments with *Valonia* made several years ago,[14] and in later measurements to be published shortly. The figure is also in good agreement with that for red blood corpuscles (0.81 μ F/cm.^{2}) published by Fricke;[15] and, less closely, with that of McClendon[16] (9 μF/cm^2.) for erythrocytes; and of Cole[17] for *Arbacia* eggs (1.02 μF/cm.^{2} at 350,000 cycles, or 18 μF/cm.^{2}, extrapolated to 1000 cycles).

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[18] Hozawa, S., *Arch. ges. Physiol.*, 1928, **219**, 111.

SUMMARY

Electrical resistance and polarization were measured during the passage of direct current across a single layer of protoplasm in the cells of *Valonia ventricosa* impaled upon capillaries. These were correlated with five stages of the P.D. existing naturally across the protoplasm, as follows:

1. A stage of shock after impalement, when the P.D. drops from 5 mv. to zero and then slowly recovers. There is very little effective resistance in the protoplasm, and polarization is slight.

2. The stage of recovery and normal P.D., with values from 8 to 25 mv. (inside positive). The average is 15 mv. At first there is little or no polarization when small potentials are applied in either direction across the protoplasm, nor when very large currents pass *outward* (from sap to sea water). But when the positive current passes inward there is a sudden response at a critical applied potential ranging from 0.5 to 2.0 volts. The resistance then apparently rises as much as 10,000 ohms in some cases, and the rise occurs more quickly in succeeding applications after the first. When the potential is removed there is a back E.M.F. displayed. Later there is also an effect of such inward currents which persists into the first succeeding outward flow, causing a brief polarization at the first application of the reverse potential. Still later this polarization occurs at every exposure, and at increasingly lower values of applied potentials. Finally there is a "constant" state reached in which the polarization occurs

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[14] Blinks, L. R., Thesis deposited in the library of Harvard University, 1926. Abstract in Summaries of Theses (1926), Cambridge, 1930, 10.

[15] Fricke, H., and Morse, S., *J. Gen. Physiol.*, 1925–26, **9**, 137.

[16] McClendon, J. F., *J. Biol. Chem.*, 1926, **69**, 733.

[17] Cole, K. S., *J. Gen. Physiol.*, 1928–29, **12**, 37.

with currents of either direction, and the apparent resistance is nearly uniform over a considerable range of applied potential.

3. A state of increased P.D.; to 100 mv. (inside positive) in artificial sap; and to 35 or 40 mv. in dilute sea water or 0.6 M $MgSO_4$. The polarization response and apparent resistance are at first about as in sea water, but later decrease.

4. A reversed P.D., to 50 mv. (outside positive) produced by a variety of causes, especially by dilute sea water, and also by large flows of current in either direction. This stage is temporary and the cells promptly recover from it. While it persists the polarization appears to be much greater to *outward* currents than to inward. This can largely be ascribed to the reduction of the reversed P.D.

5. Disappearance of P.D. caused by death, and various toxic agents. The resistance and polarization of the protoplasm are negligible.

The back E.M.F. of polarization is shown to account largely for the apparent resistance of the protoplasm. Its calculation from the observed resistance rises gives values up to 150 mv. in the early stages of recovery, and later values of 50 to 75 mv. in the "constant" state. These are compared with the back E.M.F. similarly calculated from the apparent resistance of intact cells.

The electrical capacitance of the protoplasm is shown by the time curves to be of the order of 1 microfarad per cm.^{2} of surface.

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THE SPECIFIC ACTION OF A BACTERIAL ENZYME ON PNEUMOCOCCI OF TYPE III

BY OSWALD T. AVERY AND RENÉ DUBOS

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A systematic search for enzymes capable of hydrolyzing the polysaccharides found in the capsular material of pneumococci of the various types has been carried on in this laboratory for several years. A number of enzymes from animal and plant sources, known to be active in the hydrolysis of simpler carbohydrates, were tested, but none of them were found capable of attacking the polysaccharides of pneumococcus origin. In addition, cultures of various moulds, yeasts, soil actinomycetes and bacteria, many of which were known to decompose cellulose, were tested without success. Recently, however, a bacillus has been isolated from the organic matter of soil taken from the cranberry bogs of New Jersey, which is able to split the specific capsular polysaccharide of pneumococci of Type III. The microorganism is a pleomorphic, Gram negative bacillus, motile and spore-bearing. A detailed description of the special technique employed in its isolation and cultivation, together with a more complete account of its biological characters, will be given in a subsequent publication.

From cultures of this bacillus it has been possible to extract a soluble principle which, in the absence of the living cell, decomposes this specific carbohydrate. The decomposition of the specific polysaccharide is indicated by the appearance of reducing sugars in the hydrolyzed mixtures and by the simultaneous disappearance of serological specificity. The rate of reaction and the total amount of specific substrate decomposed appear to bear a quantitative relationship to the concentration of the active principle.

The active substance present in the sterile bacterial extracts is heat labile, being destroyed by exposure to a temperature of 60° to 65° C. It is extraordinarily specific in its action against the polysaccharide of pneumococci of Type III, since the capsular carbohydrates of the specific types of Friedländer's bacillus, and even those of pneumococci of Types I and II, are unaffected. The fact that the reacting substance is a product of living cells, that it is specific and heat labile and that its action seems to conform to the laws of enzymatic reactions strongly supports the view that the active principle is of the nature of a specific enzyme.

The addition of an active extract to media does not inhibit growth or cause lysis of pneumococci; however, organisms of Type III, when grown under these conditions, are not specifically agglutinable in immune serum of the homologous type. That the *function* of elaborating the type-specific substance is not destroyed, however, is shown by the fact that pneumococci so treated continue to produce the capsular polysaccharide when transferred to a medium devoid of the active hydrolyzing agent. These two facts, namely, the decomposition of the specific carbohydrate removed from the pneumococcus cells and the hydrolysis of the specific capsular substance as rapidly as it is formed in growing cultures are evidence that the active principle is directed against this single, specific component rather than against the cell as a whole.

Previous studies on infection with pneumococci have led to the view that the invasiveness of these organisms is conditioned, in part at least, by the presence of the cell capsule. Since, early in the present work, the experimental evidence pointed to the fact that only the capsular material of the cell is vulnerable to the attack of this enzyme, it was tempting to determine whether the course of pneumococcus infection in a susceptible animal might not be favorably influenced by the injection into the animal of this specific enzyme. This possibility seemed more likely, since it was found that the activity of the enzyme *in vitro* is not inhibited or retarded by the presence of fresh animal serum. Repeated experiments with various preparations of sterile extracts containing the specific enzyme have demonstrated that the active principle has a distinct and specific protective action in mice experimentally infected with pneumococci of Type III. The protection afforded is type-specific, being effective only against pneumococci of this particular type. The protective value of the enzyme is destroyed by heating the bacterial extracts at 70° C. for 10 minutes. The capacity of any given preparation to protect animals against infection bears a definite relationship to its power to decompose the specific polysaccharide *in vitro.*

In addition to its protective action, the active principle has been found to exert a specific prophylactic and curative effect on experimental Type III pneumococcus infection in mice.

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THE BACTERIOSTATIC ACTION OF CERTAIN COMPONENTS OF COMMERCIAL PEPTONES AS AFFECTED BY CONDITIONS OF OXIDATION AND REDUCTION

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When Pneumococcus is seeded into plain broth, it is necessary to use a fairly large inoculum in order to insure the initiation of growth. The size of this inoculum increases with the amount of medium to be seeded; as a rule, 0.01 to 0.1 cc. of a plain broth culture is the minimum seeding to be used for 5 cc. of medium. These inocula contain at least 1,000,000 to 10,000,000 cells. What are the factors which make such a large number of cells necessary? Under what conditions would one single cell grow?

An answer to these questions will probably result in a better understanding of the physiology of the pneumococcus cell. But in addition, the preparation of an "ideal" medium (and no medium is "ideal" which does not allow the growth of a single cell) would be of great value in the study *in vitro* of the bacteriostatic and bactericidal properties of the body fluids of immunized and naturally resistant animals. However, the present work was undertaken to meet a purely practical need. During the past few years, considerable difficulties have often been encountered in the preparation of media suitable for the growth of Pneumococcus. Many lots of broth were found to be unable to support growth even when large inocula were used. Yet it has been observed that when various lots of media are prepared from the same meat infusion, but with the addition of different brands of peptones, these various lots differ in their ability to support growth of Pneumococcus, depending upon the kind of peptone employed; in all cases, growth would occur, even with very small inocula, provided a sufficient amount of neutral cysteine or thioglycollic acid was added. It seems therefore that the inability to support growth depends upon some bacteriostatic property of the peptone and that this effect may be overcome by the addition of reduced thioacids.

It is known in a general way that the living cell can greatly affect the conditions of its environment. In particular, it may be recalled that pneumococcus cells acting on certain metabolites bring about the formation of actively reducing substances (1, 2). If, however, *very small inocula* be employed, the influence of the reducing action of the bacteria can be considered negligible. Advantage may be taken of this observation in devising an experimental method for the study of those properties of the medium which affect the initiation of growth.

Experimental Methods

Cultures.—All experiments were carried out with a strain of Type II Pneumococcus (D/39/79) and an R culture derived from Type II (D/39/R). Occasionally, control experiments were carried out with strains of Type I and Type III Pneumococcus. The results being always substantially the same, only the data obtained with the Type II strain are presented in this article.

Unless otherwise stated, the inoculum consisted of 0.0001 cc. of a 12-hour culture in plain broth.

Ingredients Used in the Preparation of Media.—The beef infusion was prepared as usual, enriched with 0.03 per cent glucose, adjusted to pH 7.8 and autoclaved.

"Tryptophane broth" is the commercial name of a dehydrated preparation of casein hydrolysed with acid. Solutions of 1 per cent of this dehydrated medium were enriched with 0.03 per cent glucose, adjusted to pH 7.8, and autoclaved.

Peptone solutions employed were of 10 per cent concentration; they were adjusted to pH 7.8 and autoclaved.

The cysteine and thioglycollic acid were autoclaved in neutral 1 per cent solutions and kept under vaseline seal. The results reported in this paper were obtained with thioglycollic acid; no difference could be noticed between the action of the two thiol compounds.

The oxidized thiol compound used was a product obtained by the auto-oxidation of thioglycollic acid (Eastman), in the presence of air, with sodium nitroprusside as a catalyst (in dilute ammonia solution); this product will be referred to as "oxidized thioglycollic acid" although its exact nature is not known since it was not obtained in crystalline form.

The Bacteriostatic Action of Peptones and the Possibility of Correcting It by Means of Reduced Thiol Groups

It has been shown previously (3) that the growth of very small inocula of Pneumococcus in plain broth may occur when cysteine hydrochloride or thioglycollic acid is added to the medium. Experiment 1 gives the minimum concentration of thioglycollic acid which is necessary for growth to occur.

Experiment 1—Varying amounts of thioglycollic acid were added to different portions of the same lot of plain broth (prepared 1 week previous to use), and tubes containing 5 cc. of these media received inocula varying from 0.1 cc. to 0.0000001 cc. of a plain broth culture of Pneumococcus. The cultures were incubated at 37°C. and final readings made after 48 hours incubation.

The results of Experiment 1 illustrate the fact that, in the plain broth under consideration, an inoculum of 0.1 cc. is necessary to obtain the growth of Pneumococcus. However, growth will occur even if the inoculum be only 0.000001 cc. in the presence of 0.005 per cent thioglycollic acid. It is apparent therefore that plain broth exerts a bacteriostatic effect on Pneumococcus and that a concentration of 0.005 per cent thioglycollic acid can overcome this effect.

TABLE I

The Influence of Thioglycollic Acid on the Growth of Small Inocula of Pneumococcus in Plain Broth (Peptone A)

Final concentration of thioglycollic acid (in per cent)	Inoculum (cc. of pneumococcus culture)							
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
0	+	−	−	−	−	−	−	−
0.001	+	+	+	−	−	−	−	−
0.002	+	+	+	−	−	−	−	−
0.005	+	+	+	+	+	+	+	−
0.01	+	+	+	+	+	+	+	−
0.02	+	+	+	+	+	+	+	+
0.05	+	+	+	+	+	+	+	−

The signs + and − refer to the presence or absence of growth after 48 hours incubation.

The object of the next experiment was to determine whether the minimum concentration of thioglycollic acid required for the growth of small inocula of Pneumococcus would be affected by the kind of peptone used in the preparation of the medium.

Experiment 2.—Media were prepared from the same meat infusion but with different peptones, 2 per cent concentrations being used of either one of the four different commercial preparations of peptone designated as A, B, C, and D. Amounts of 5 cc. of these media were treated with different concentrations of thioglycollic acid, inoculated with 0.0001 cc. of pneumococcus culture and incubated at 37°C. Table II shows the results after 24 hours incubation.

The results of Experiment 2 indicate that the bacteriostatic effect of the broth varies greatly with the different peptones used in the preparation of the medium; this is evidence that a part, at least, of this bacteriostatic power is to be traced to the peptone.

TABLE II

The Effect of Using Different Commercial Peptones on the Growth of Pneumococcus in the Presence of Varying Concentration of Thioglycollic acid

Medium: meat infusion with	Final concentration of thioglycollic acid (in per cent)							
	0	0.0001	0.002	0.004	0.006	0.008	0.01	0.02
Peptone A	−	−	−	−	−	−	−	+
" B	−	−	−	+	+	+	+	+
" C	−	−	−	+	+	+	+	+
" D	−	−	+	+	+	+	+	+

The signs + and − refer to the presence or absence of growth after 24 hours incubation.

The results also suggest that the amount of thioglycollic acid necessary for growth to occur might be used as a measure of the bacteriostatic action of the peptone.

The experiments that follow were all performed with peptone A which appears to have the most pronounced bacteriostatic power.

In order to demonstrate that thioglycollic acid can be used as a measure of the bacteriostatic action of the peptone, an attempt was made to establish a quantitative relationship between the amount of peptone added to the meat infusion and the concentration of thioglycollic acid required to overcome the bacteriostatic action of such media.

Experiment 3.—Media were prepared from the same meat infusion, but with concentrations of peptone (brand A) varying from 0.5 per cent to 4 per cent; these media were treated with varying concentrations of thioglycollic acid, inoculated with 0.0001 cc. of pneumococcus culture (per 5 cc. of medium) and incubated at 37°C.

The results of Experiment 3 indicate that as the concentration of the peptone in the medium increases, the concentration of thioglycollic acid necessary for growth also increases. A more exact relationship will be obtained when, instead of the whole peptone, we use the isolated fraction responsible for the bacteriostatic action (see Experiment 8). This indicates that thioglycollic acid can be used to titrate the bacteriostatic power of the peptone.

TABLE III

The Relation between the Concentration of Peptone (Brand A) Used in the Preparation of the Broth, and the Concentration of Thioglycollic Acid Required for the Growth of Small Inocula of Pneumococcus

Concentration of peptone A (in per cent)	Final concentration of thioglycollic acid (in per cent)						
	0	0.001	0.002	0.004	0.006	0.008	0.01
4	–	–	–	–	–	–	+
2	–	–	–	–	+	+	+
1	–	–	–	+	+	+	+
0.5	–	+	+	+	+	+	+

The signs + and – refer to the presence and absence of growth after 48 hours incubation.

It has been suggested previously (3) that cysteine owes its beneficial action on the growth of Pneumococcus to its reducing power.

This suggestion will be confirmed by comparing the effect of the oxidized and reduced forms of thioglycollic acid on the growth of Pneumococcus in plain broth.

Experiment 4.—Different portions of plain broth prepared with 1 per cent of peptone A were treated with "reduced" and "oxidized thioglycollic acid." Amounts of 5 cc. of these media were seeded with inocula ranging from 0.1 to 0.00001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 4 indicate that, following oxidation, thioglycollic acid becomes unable to correct the bacteriostatic effect of the peptone; it may be noted, however, that "oxidized thioglycollic acid" is in no way toxic, as is shown by comparison with the control.

Thioglycollic acid therefore appears to owe its activity to the presence of reduced thiol groups in the molecule.

The Existence in Peptone A of Substances Which Are Bacteriostatic under Oxidizing Conditions and Not under Reducing Conditions

It has just been shown that the bacteriostatic power of peptone can be corrected by treatment with reduced thiol groups. It is also greatly affected by the conditions of oxidation and reduction under which the peptone solutions are kept. This can be shown by the five following examples.

Experiment 5.—a, b, c. Test tubes containing 4.5 cc. of meat infusion received 0.5 cc. of a 10 per cent peptone solution (at pH 7.8) which had been autoclaved.

In preparation *a*, the peptone solution was used *immediately after autoclaving*; in preparation *b*, it had been kept for 48 hours at room temperature under *aerobic conditions*; in preparation *c* it had been kept for 48 hours at room temperature under *vaseline seal.*

d. The peptone solution which had been kept aerobically for 48 hours (preparation *b*) was added to meat infusion and the mixture kept under vaseline seal for 2 weeks at room temperature.

e. A similar mixture (preparation *d*) which had been kept under vaseline seal for 2 weeks was now transferred to a flask and kept aerobically for 2 days.

Test tubes containing 5 cc. of the media (prepared as described under *a*, *b*, *c*, *d*, and *e*) were inoculated with 0.0001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 5 prove that peptone A used immediately after autoclaving, (preparation *a*), or kept constantly under anaerobic conditions (preparation *c*), exhibits only a very slight bacteriostatic power. On the other hand, the same peptone solution becomes markedly bacteriostatic following exposure to the air (preparation *b*). A peptone solution that has become bacteriostatic following exposure to the air loses its bacteriostatic effect by incubation under vaseline seal in the presence of meat infusion (preparation *d*); but again becomes bacteriostatic following reexposure to the air.

These facts, as well as the beneficial action of reduced thiol groups, point to the existence in peptone A of a substance which is bacteriostatic in the oxidized form, and not in the reduced form; it can be oxidized by atmospheric oxygen and reduced by -SH groups and meat infusion; this oxidation and reduction appears to be reversible.

In the course of these experiments, it was observed that the color of the peptone A varies with the different conditions under which the solution is kept. The solution is dark brown when taken out of the autoclave or when kept under vaseline seal. The color turns yellow on the contrary under aerobic conditions. The yellow color could be changed again to the darker by treatment with thioglycollic acid or cysteine. In other words, the darker color corresponds to the reduced non-bacteriostatic form of the peptone solution, whereas the yellow color corresponds to the oxidizèd-bacteriostatic form.

TABLE IV

The Growth of Pneumococcus in Plain Broth (Peptone A) in the Presence of Reduced and Oxidized Thioglycollic Acid

Medium	Inoculum (cc.)				
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Plain broth	+	+	−	−	−
" " + 0.01 per cent reduced thioglycollic acid	+	+	+	+	+
Plain broth + 0.01 per cent oxidized thioglycollic acid	+	+	−	−	−

The signs + and − refer to the presence or absence of growth after 48 hours incubation.

TABLE V

Effect of Maintaining Peptone Solutions under Oxidizing and Reducing Conditions on the Bacteriostatic Effect of Media Prepared with Them

Media*	Final concentration of thioglycollic acid (in per cent)						
	0	0.0005	0.001	0.002	0.003	0.004	0.005
Preparation *a*	−	+	+	+	+	+	+
" *b*	−	−	−	−	−	−	+
" *c*	−	−	+	+	+	+	+
" *d*	−	+	+	+	+	+	+
" *e*	−	−	−	−	−	+	+

* The media referred to as preparations *a*, *b*, *c*, *d*, and *e* are described in the text.

The signs + and − refer to the presence or absence of growth after 48 hours incubation.

It was tempting to suppose that the chromogenic fraction responsible for the change in color was associated with the bacteriostatic agent, and an attempt was made to fractionate peptone A on the basis of this assumption.

Isolation of the Fraction of Peptone A Containing the Bacteriostatic Substance

Different agents were used in an attempt to isolate the chromogenic fractions of peptone A (extraction and precipitation with HCl, NaOH, $(NH_4)_2SO_4$, copper salts, trichloracetic acid, alcohol, acetone, ether). The best results were obtained with the following procedure.

200 gm. of peptone were dissolved in 1000 cc. of tap water and autoclaved. On the following day, the solution was treated with 15 cc. of concentrated hydrochloric acid. A heavy precipitate formed rapidly and was separated by filtration or centrifugalization. This dark green precipitate will be designated as fraction 1.

The filtrate (or supernatant) was made alkaline with 20 cc. of 40 per cent sodium hydroxide and 1000 cc. of acetone was added to the solution. A lighter precipitate of a greyish color separated and was designated as fraction 2.

A further amount of 1050 cc. acetone was added to the supernatant; a dark yellow (resin-like) material was seen to run down the walls of the container and could be separated in a separatory funnel. This will be designated as fraction 3.

After removal of this "yellow fraction," 3 liters of acetone were again added and a heavy white floculum was formed. This will be designated as fraction 4.

The remaining acetone solution was still slightly pigmented yellow; although it was found possible to separate this new pigment in very alkaline solution, no attempt was made to separate it on a large scale. The bulk of the solution was evaporated down and designated as fraction 5.

Each one of the 5 fractions was made up to 2 liters (corresponding therefore to a 10 per cent solution of peptone) and adjusted to pH 7.8. Fraction 1 appeared then as a dark green solution somewhat cloudy. Fraction 2 gave a greyish suspension, little soluble in water, and which settled somewhat on standing. Fraction 3 gave a perfectly clear, but very dark yellow solution. Fraction 4 gave a limpid solution with a very slight yellow tinge (due probably to a contamination with the preceding fraction). Fraction 5 gave a clear, slightly yellow solution.

5 cc. amounts of these different fractions were treated with 0.5 cc. of 1 per cent thioglycollic acid. Within half an hour (at room temperature), fraction 1 began to change color, turning first to a brown-yellow, then to a dark brownish color. The other fractions remained unchanged. It is therefore apparent that fraction 1 is responsible for the change in color observed in the peptone following oxidation and reduction.

It was also observed that following the addition of 0.1 cc. of a 3 per cent solution of potassium cyanide, fraction 1 turned whitish while the other fractions were not affected.

The object of this experiment was to determine which of the fractions were responsible for the bacteriostatic action of peptone A.

Experiment 6.—Media were prepared by the addition of 0.5 cc. of the different fractions and of whole peptone (10 per cent solution) to 4.5 cc. of meat infusion. These media were treated with different concentration of thioglycollic acid and inoculated with 0.0001 cc. of a pneumococcus culture.

TABLE VI

The Bacteriostatic Effect on Pneumococcus of Media Prepared by the Addition to Meat Infusion of Different Fractions of Peptone A

Medium containing	Final concentration of thioglycollic acid (per cent)					
	0	0.001	0.002	0.003	0.004	0.005
Fraction 1	−	−	−	−	−	−
" 2	+	+	+	+	+	+
" 3	+	+	+	+	+	+
" 4	+	+	+	+	+	+
" 5	−	−	−	−	−	−
Whole peptone	−	−	−	−	+	+

The signs + and − refer to the presence or absence of growth after 48 hours incubation.

It is apparent from the results of Experiment 6 that fractions 2, 3 and 4 of peptone A can support the growth of Pneumococcus when added to meat infusion—*and do not exhibit any bacteriostatic effect.* The absence of growth with fractions 1 and 5 may be attributed to the lack of some factor essential to growth or to the bacteriostatic action of these fractions. This question is the subject of the following experiment.

Experiment 7.—A medium was prepared by the addition of 15 cc. of fraction 4 to 135 cc. of meat infusion. To tubes containing 5 cc. amounts of this medium was added 0.5 cc. of peptone solution (10 per cent), of fraction 1 or of fraction 5 along with different concentrations of thioglycollic acid. The tubes were then inoculated with 0.0001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 7 demonstrate that practically all the bacteriostatic action of the peptone is due to fraction 1 and only to it.

In all these experiments, thioglycollic has been used as a reagent for titrating the bacteriostatic action of the materials under consideration. In fact, it has been found in Experiment 3 that the amount of thioglycollic acid required to initiate growth of small inocula increases with the concentration of peptone in the medium. The subject of Experiment 8 was to determine whether a more exact relationship could be obtained between the amount of fraction 1 added to the medium and that of thioglycollic acid required to overcome its bacteriostatic action.

Experiment 8.—Attention must be called to two modifications introduced in the experimental procedure. Meat infusion is known to possess a high reducing capacity and it is likely that it would thus interfere with the establishment of the quantitative relationship between fraction 1 and thioglycollic acid. Tryptophane broth on the contrary has very little reducing capacity; it can support the growth of Pneumococcus when added to fraction 3 of peptone. It was therefore used instead of meat infusion in this experiment.

TABLE VII

The Bacteriostatic Action of Fraction 1 of Peptone A

Medium: meat infusion with	Concentration of thioglycollic acid (per cent)						
	0	0.0004	0.002	0.005	0.01	0.02	0.05
Fraction 4	+	+	+	+	+	+	+
" 4 + fraction 1	−	−	−	+	+	+	+
" 4 + fraction 5	−	+	+	+	+	+	+
Whole peptone	−	−	−	+	+	+	+

The signs + and − refer to the presence or absence of growth after 48 hours incubation.

The other modification was devised in view of the fact that the fraction 1, after being reduced by thioglycollic acid, may be reoxidized by atmospheric oxygen. This was prevented by incubating the culture under vaseline seal.

Varying amounts of fraction 1 were added to test tubes containing 4.5 cc. of medium consisting of tryptophane broth and fraction 3; these media were treated with different concentrations of thioglycollic acid, inoculated with 0.0001 cc. of pneumococcus culture and incubated under vaseline seal.

Table VIII brings out the close proportionality between the concentration of fraction 1 in the medium and the amount of thioglycollic

TABLE VIII

Titration of the Bacteriostatic Fraction of Peptone A by Means of Thioglycollic Acid

Amount of fraction 1 added to the medium (in per cent)	Final concentration of thioglycollic acid (in per cent)						
	0	0.0005	0.001	0.002	0.005	0.01	0.02
0	+	+	+	+	+	+	+
0.2	−	+	+	+	+	+	+
0.5	−	−	−	−	+	+	+
1.0	−	−	−	−	−	+	+
2.0	−	−	−	−	−	−	+

The signs + and − refer to the presence or absence of growth after 48 hours incubation.

TABLE IX

The Effect of Potassium Cyanide on the Bacteriostatic Properties of the Fraction 1 of Peptone A

Medium: meat infusion with	Final concentration of thioglycollic acid (in per cent)				
	0.0005	0.002	0.005	0.01	0.02
Fraction 4	+	+	+	+	+
KCN + fraction 4	+	+	+	+	+
Fraction 1 + fraction 4	−	−	−	+	+
KCN + fraction 1 + fraction 4	+	+	+	+	+

TABLE X

The Growth of Pneumococcus in Media Prepared with Whole Peptone and "Purified Peptone"

Materials added to meat infusion	Final concentration of thioglycollic acid (in per cent)						
	0	0.0001	0.0002	0.005	0.01	0.02	0.05
Whole peptone	−	−	−	−	−	−	+
Purified peptone	−	+	+	+	+	+	+
Fraction 1	−	−	−	−	−	−	−
Purified peptone + fraction 1	−	−	−	−	−	−	−

The signs + and − refer to the presence or absence of growth after 48 hours incubation.

acid required for growth to develop. This relation, however, does not apply when very small concentrations are used.

It has been mentioned previously that, when treated with potassium cyanide, the green pigment rapidly becomes whitish. Experiment 9 demonstrates that fraction 1 loses at the same time its bacteriostatic properties.

Experiment 9.—A medium was prepared by the addition of 10 per cent of fraction 4 to meat infusion. Test tubes containing 5 cc. of this medium received 0.5 cc. of fraction 1, or 0.02 cc. of 3 per cent KCN, or both. They were then treated with different concentrations of thioglycollic acid, inoculated with 0.0001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 9 show that the bacteriostatic properties of fraction 1 can be removed by treatment with potassium cyanide.

The preceding experiments suggest that a solution of peptone A from which the green bacteriostatic fraction has been removed should supply a satisfactory medium when added to meat infusion.

Experiment 10.—A 10 per cent solution of peptone A was treated with the proper amount of concentrated HCl to precipitate fraction 1. This fraction was then made up to the original volume of the peptone solution and adjusted to pH 7.8.

The peptone solution from which fraction 1 had been removed appeared as a yellow fluid which was also adjusted to pH 7.8. This second fraction will be designated as "purified peptone."

These two solutions were added in amounts of 0.5 cc. to 4.5 cc. of meat infusion as described in Table X and the media treated with different concentrations of thioglycollic acid. The tubes were inoculated as usual with 0.0001 cc. of pneumococcus culture and incubated at 37°C.

The results of Experiment 10 demonstrate that by acid precipitation, one can remove almost completely the bacteriostatic properties of a solution of the commercial peptone A.

DISCUSSION

The facts recorded in this article seem to warrant the conclusion that there exists in commercial peptones a substance (or substances) which may exert a bacteriostatic action on the growth of certain organisms. Its action in plain broth is well demonstrated by the lack of growth of small inocula of Pneumococcus under ordinary conditions. When, on the contrary, the same medium is prepared with peptones from which the bacteriostatic substance has been removed, or when the bacteriostatic effect has been corrected by reduction, inocula consisting of only one or a very few cells are sufficient to initiate growth.

The bacteriostatic substance is heat stable and, in the brand of peptone which has been studied, it is associated with a certain fraction obtained by acid precipitation; this fraction is pigmented, and changes color upon oxidation and reduction; the bacteriostatic property of the same peptone fraction is also controlled by conditions of oxidation and reduction. But we have no other evidence that the chromogenic substance and the bacteriostatic substance are one and the same thing.

Concerning the mode of action of this substance, we have at least an excellent clue; it is bacteriostatic only under oxidizing conditions, not at all under reducing conditions. In this respect, it behaves similarly to some dyes previously studied (indophenols, methylene blue, gallocyanine,[1] which are bacteriostatic in the oxidized form, but not in the reduced form (3).

Methylene blue has been found to be bacteriostatic for Pneumococcus in concentrations as low as 0.00003 molar. There is as yet no direct way of measuring at what minimum concentration the bacteriostatic substance of peptone exerts its activity, but some conception can be gained by an indirect method. As already shown, there exists a certain quantitative relationship between the amount of bacteriostatic substance and the concentration of thioglycollic acid which can overcome its action. In certain experiments, it has been sufficient to add as little as 0.0003 per cent of this thiol compound to allow the growth of an inoculum which would not have otherwise developed; the molecular weight of thioglycollic acid being 92, one sees that the molar concentration is of the same order as that found in the case of methylene blue. Expressed in other terms, this means that the addition of 0.000003 gm. of thioglycollic acid per cubic centimeter of medium may determine whether growth will take place or not. These concentrations are of the order found in catalytic reactions, and one may well wonder whether the bacteriostatic substance of the peptone does not act as anticatalyst in some phase of the metabolism of the cell.

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[1] The data concerning the effect of gallocyanine have not yet been published.

Whatever the mechanism of its action may be, this substance is active in extremely small amounts. We have evidence of organisms which are much more sensitive to it than the ordinary strains of Pneumococcus. It will be worth while to study the rôle of such substances in determining whether a bacterial species is "aerobic" or "anaerobic," whether it is "easy" or "hard" to grow.

The growth of large inocula of Pneumococcus in plain broth, as compared with the lack of development of smaller inocula, is now readily understood. It has been shown (1, 2) that pneumococcus cells, when in the presence of certain metabolites, give rise to a very actively reducing system; this system is able to reduce (and therefore inactivate) the bacteriostatic substance of the peptone and thus renders the medium suitable for the development of growth.

The significance of the facts presented in this article may well transcend their importance for the growth of Pneumococcus in artificial media. One wonders whether there do not exist in tissues mechanisms similar to the one described, which would render impossible the multiplication of bacteria under normal conditions of oxidation. If, however, local reducing conditions were to develop, is it not possible that bacterial invasion of the tissues would then be facilitated? It is well to remember, in this respect, that the peptones under consideration were probably prepared from materials of animal origin. In fact, tryptic digest broth and peptic digest broth prepared from beef were also found to exhibit some bacteriostatic properties, but this action was demonstrable only under aerobic conditions.

SUMMARY

There are present in commercial peptones substances which exhibit bacteriostatic properties for certain bacterial species. These substances are bacteriostatic in the oxidized form, but not in the reduced form. Their bacteriostatic action can be overcome, and their concentration titrated, by the addition of reduced thiol compounds to the media in which they are present.

Different brands of peptone differ greatly in the amount of bacteriostatic substances they contain; these differences account, in part at

least, for the fact that media prepared from the same meat infusion, but with different kinds of peptone, vary in their ability to support bacterial growth.

The bacteriostatic fraction of a certain peptone solution can be completely removed by precipitation with acid and acetone. A peptone which has thus been purified becomes capable of supporting the growth of very small inocula of Pneumococcus.

The significance of the sensitiveness of certain bacterial species to substances which are bacteriostatic in the oxidized but not in the reduced form is considered with reference to (a) the mechanism of bacteriostasis, (b) the growth of bacterial species in artificial media, (c) the problem of infection.

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THE DISTRIBUTION OF FRIEDLÄNDER'S BACILLI OF DIFFERENT TYPES

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In 1926 (1), a number of strains of Friedländer's bacillus were classified by the immunological reactions of agglutination, agglutinin adsorption and passive protection against infection, into three specific types, which were arbitrarily designated as Types A, B, and C. The strains falling into the specific types comprised the great majority of those studied, and the remaining cultures which were found to differ serologically from each other and from the specific types were included in a heterogeneous group called Group X. The distinctions in type were referable to the presence of a specific carbohydrate in the capsular material. The carbohydrate (2), or soluble specific substance, was shown to be chemically different for each of the three types.

Cultures of Friedländer's bacillus have been collected in the meantime in order to study the relative frequency and distribution of the organism of various immunological types.

EXPERIMENTAL

In the present survey a total of 80 strains have been isolated from a variety of disease conditions; in most instances the organisms were considered to be the inciting agent of the disease, in other cases secondary invaders. All the organisms studied were encapsulated, non-motile, non-spore-bearing, Gram negative rods with the cultural characteristics usually attributed to Friedländer's bacillus. In each instance the serological type was determined by the agglutination of live young cultures (4 to 6 hours) in type-specific sera.

The fermentation of carbohydrates by the different strains was also studied to determine possible serological and biochemical relationships.

The Frequency of Occurrence of Friedländer's Bacilli of the Different Specific Types.—In Table I is given the relative number of strains of the different types isolated from disease. It is seen that of the 80 strains, about 75 per cent fall in one or other of the three specific types. To Type A, belong 42 of the strains (52 per cent); to Type B, 12 (15 per cent); to Type C, 7 (9 per cent). Group X includes 19 (24 per cent) of the cultures. It is interesting to compare these figures with those obtained from the original study (1) of 30 strains. At that time, it was found that 50 per cent of the strains were of Type A, 20 per cent of Type B, 10 per cent of Type C, and 20 per cent belonged in Group X.

The Incidence of the Various Specific Types in Different Diseases.—This is shown in Table II. Most of the strains studied were isolated from cases of pneumonia in man. Of the strains isolated from this source about 75 per cent were of Type A. In the original study (1) it was found that about 70 per cent of the total strains associated with pneumonia belonged to Type A. Most of the Type A strains were isolated from human cases where the majority of Type B strains were isolated from animals. The organisms of Group X were obtained from a variety of conditions in animals and man.

TABLE I

The Distribution of Specific Types of Friedländer's Bacillus

Total number of strains studied	Distribution by types			Group X
	Type A	Type B	Type C	
80	42	12	7	19

The Specific Precipitin Reaction in the Urine in Pneumonia.—In a preceding paper (3) it was shown that rabbits experimentally infected with Friedländer's bacillus eliminate the soluble specific substance through the kidney, and that the urine of infected animals may give a precipitation reaction in the corresponding type-specific serum. Blake (4) first demonstrated the presence of a positive precipitin reaction in a case of pneumonia in man due to Friedländer's bacilli. Recently in a case of pneumonia in the Hospital of The Rockefeller Institute, due to Friedländer's bacillus, the opportunity was offered for studying the type-specific precipitin reaction in the urine. On the day of admission, which was the second day of the disease, Friedländer's bacillus Type A was isolated both from the blood and sputum, and the presence of the soluble specific substance was demonstrated in the urine by a type-specific precipitation reaction which was positive even when the urine was diluted sixteen times. On the following day the disease terminated fatally and this day the precipitation reaction was positive when the urine was diluted 1:64.

TABLE II

The Incidence of the Various Types of Friedländer's Bacillus in Different Diseases

Type	Human pneumonia	Throat	Liver abscess	Animal source	Miscellaneous
Type A (42 strains)	33	3	1	Guinea pig—2 (not pneumonia) (fatal abscess)	Cystitis—1 Adenoid tissue—2
Type B (12 strains)	2	1	1	Guinea-pig—5 (pneumonia) Mare—2 (G. U. infections)	Feces—1 (colitis)
Type C (7 strains)	3	0	0		Antrum—1 (sinusitis) Nose—1 (sinusitis) Unknown—2
Group X (19 strains)	7	2	1	Guinea-pigs—2 (not pneumonia) (fatal abscess)	Sputum—2 (not pneumonia) Cystitis 3 Lung abscess—1 Feces—1 (Pellagra)

The Fermentation of Carbohydrates by Friedländer's Bacilli of the Different Types.—The fermentation reactions of bacteria of the *Encapsulatus* group have been studied by a number of workers, but the results have been contradictory and confusing. While certain authors have found that Friedländer's bacillus ferments lactose with the formation of acid and gas (5, 6, 7, 8) or of acid alone (9, 10, 11, 12), other writers (13, 14, 15) have observed that it does not ferment lactose. Still other authors (16, 17, 18, 19) state that different strains may vary in their ability to ferment lactose, and it is the opinion of these authors that the fermentation reactions of the bacteria of the *Encapsulatus* group are too inconstant to be of value in classification or identification.

TABLE III

The Fermentation Reactions of Friedländer's Bacillus of the Different Specific Types

Friedländer bacilli		Reactions in				
Type	Number of strains	Dextrose	Lactose	Sucrose	Maltose	Mannitol
A	30	AG	AG	AG	AG	AG
B	8					
C	3					
Group X	8					
A	9	AG	Negative	AG	AG	AG
B	None					
C	1					
Group X	1					
A	2	A	A	A	A	A
B	2					
C	None					
X	5					
A	4	A	Negative	A	A	A
B	None					
C	None					
X	None					
X	1	AG	AG	Negative	AG	Negative
X	1	A	Negative	Negative	Negative	Negative
X	1	A	Negative	Negative	AG	Negative

A indicates presence of acid.
AG indicates presence of acid and gas.
Negative indicates absence of acid and gas.

It seemed, however, important to study again this question with reference to the specific types of organism employed in making the tests.

Five carbohydrates (dextrose, lactose, sucrose, maltose and mannitol) were employed, since earlier observations indicated that these five offered the greatest possibilities for use in differentiation. Samples of meat extract broth containing 1 per cent of the respective carbohydrates were inoculated, and the final readings were made after an incubation period of 7 days.

The results of this study are given in Table III. It is seen that the majority of the strains ferment all 5 carbohydrates with the formation of acid and gas. There is, however, a great variability in the fermentative activities of the different strains, and it is obvious that a classification of Friedländer's bacilli on the basis of fermentation activity is of little value. Furthermore, the fermentation reactions do not aid in the identification of the specific types. It is interesting to note, nevertheless, that of 15 strains which form neither acid nor gas in lactose, 13 are of Type A. In other words, the inability to ferment lactose is most characteristic of Type A cultures. Of further interest is the fact that the strains of Group X show the greatest variability in the fermentation of carbohydrates.

DISCUSSION

The present communication records the distribution of Friedländer's bacilli of the different specific types in disease. It was found that most of the Type A strains are obtained from human cases and the majority of them from cases of pneumonia. On the other hand, the greatest number of strains of Type B are derived from animal sources, while Group X contains organisms isolated from the greatest variety of diseases. In this connection, it should be pointed out that in confirming the existence of specific types among the Friedländer bacilli, Edwards (18, 19) characterizes his Type I (corresponding to Type B) as the animal type, and Type II (corresponding to Type A) as the human type. Edwards (19) also shows that certain strains of *Bacterium aerogenes* are serologically identical with Friedländer's bacilli. This is not surprising, however, when it is recalled that Avery, Heidelberger and Goebel (20) had previously demonstrated the immunological similarity of Type B Friedländer's bacillus to the more widely remote species, Pneumococcus Type II.

It has been possible to confirm Blake's observations on the occurrence of a specific precipitin reaction in the urine during the course of pneumonia due to Friedländer's bacillus. Dochez and Avery (21), also demonstrated during their original studies on the soluble specific substance of Pneumococcus that the specific urine precipitation tests in immune sera offered a procedure of diagnostic as well as prognostic importance.

A study of the fermentation reactions of Friedländer's bacillus confirms an earlier opinion (17) that a classification based on these reactions possesses little value. Fitzgerald (16) previously and Edwards (18, 19) more recently reached similar conclusions. In the present study, it was not possible to show any constant relationship between serological reactions and biochemical activity as measured by the fermentation of carbohydrates, although it was found that among the strains of all types there is a much greater tendency for Type A strains not to form acid or gas from lactose.

SUMMARY AND CONCLUSIONS

In a study of the distribution of the specific types of Friedländer's bacillus, it is shown that: (1) Of 80 strains 52 per cent belong to Type A, 15 per cent to Type B, 9 per cent to Type C, and 24 per cent to Group X. (2) Type A contains for the most part strains derived from diseases of man and more than 70 per cent are associated with pneumonia in man. (3) Type B includes the greatest number of strains from animal sources, while the heterogeneous strains comprising Group X come from the greatest variety of diseases.

It was demonstrated that in a patient suffering with pneumonia due to Friedländer's bacillus (Type A), a specific precipitin reaction of the urine occurred in the corresponding (Type A) immune serum.

A study of the sugar fermentation reactions of Friedländer's bacillus shows that (1) there is no correlation between serological type and fermentative activity; (2) the fermentation reactions are variable and therefore not reliable for distinguishing Friedländer's bacillus from closely allied organisms; (3) the strains of Group X show the greatest variation in fermentation, and (4) of 15 strains unable to ferment lactose, 13 belong to Type A.

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SEROLOGICAL REACTIONS IN PNEUMONIA WITH A NON-PROTEIN SOMATIC FRACTION OF PNEUMOCOCCUS*

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It has been shown (1) that pneumococci contain two constituents which are chemically and antigenically distinct. One of these, the type-specific component, is a complex polysaccharide, predominantly present in the capsule of the organism; the other, a substance common to the pneumococcus species, is the so-called nucleoprotein, contained for the most part in the body of the cell. That these two chemically distinct fractions are responsible for the production of two qualitatively different antibodies has been demonstrated (1, 2).

The present report is based upon observations made with a third fraction derived from pneumococci and chemically distinct from both type-specific capsular polysaccharide and non-type-specific somatic nucleoprotein. For purposes of reference this substance is designated Fraction C. The chemical nature of Fraction C and the method of purification together with certain experimental observations are presented in a separate communication (3). In this report it is sufficient to state that Fraction C is a non-protein material of somatic origin and appears to be a carbohydrate common to the pneumococcus species. Although final proof of its exact nature rests upon chemical analysis, nevertheless convincing evidence of the separate identity of Fraction C is brought out by the serological reactions to be described.

Material and Methods

Preparation of Fraction C.—The material employed in the serological tests was derived from a degraded, non-type-specific R strain of Pneumococcus. A strain of this character was employed in order to minimize the presence of type-specific carbohydrate. Fraction C was obtained in the following manner: The organisms contained in several liters of full-grown broth culture of an R strain were centrifuged and resuspended in normal salt solution in 60 to 100-fold concentration. The bacteria were then frozen and thawed several times until dissolution had been effected. 0.3 cc. to 0.5 cc. of normal acetic acid was added and the solution boiled for 8 to 10 minutes. The heavy coagulum thus formed was removed by centrifugation. Acidulation and boiling were repeated to insure removal of all acid and heat precipitable material. The final water-clear supernatant fluid, neutralized with normal NaOH, contained Fraction C. Material prepared according to this simple procedure was comparable in reactivity and specificity to more highly purified preparations. Consequently, further steps in purification were not, as a rule, carried out. Some lots, however, were treated by repeated precipitation with 4 to 5 volumes of 95 per cent alcohol.

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In the charts the reaction is represented as occurring in dilutions of C substance as high as 1 to 640,000. This figure is only approximate since a quantitative estimation was not made on all lots of material. However, accurate measurement was made with one sample; using it as a standard some of the sera were re-tested and found to react with a 1 to 1,000,000 dilution. Consequently the approximate figures given are believed to be conservative estimates.

The chemical fractionation of numerous bacteria by procedures similar in many respects to that just described, has been reported by others. Pick (4) derived from typhoid bacilli an apparently non-protein substance which reacted especially in immune serum. Zinsser (5) and Zinsser and Parker (6) isolated so-called residue antigens from tubercle bacilli, pneumococci, staphylococci, influenza bacilli, and typhoid bacilli. Laidlaw and Dudley (7) obtained a carbohydrate from tubercle bacilli. Furth and Landsteiner (8) obtained from typhoid and paratyphoid B cultures three fractions, one of which appeared to be non-protein. Day (9) derived from staphylococci an "antigenic specific substance." Lancefield (10) separated a somatic carbohydrate from hemolytic streptococci which she designated Fraction C and which is comparable in many respects to the pneumococcus Fraction C.

Pneumococcus Type-Specific Polysaccharides.—The preparations employed were made according to the method described by Heidelberger and Avery (11).

Pneumococcus Nucleoprotein.—The material was made by precipitation with acetic acid in the cold as described by Avery and Morgan (12).

Precipitin Tests.—0.2 cc. test serum was diluted to 0.5 cc. with physiological salt solution; to this was added an equal volume of varying dilutions of precipitinogens. Final readings were made after the mixtures had been kept in water bath at 37° for 2 hours and over night in the ice box.

Agglutination Tests.—0.5 cc. of varying dilutions of serum was added to 0.5 cc. of heat-killed pneumococci suspended in physiological salt solution. The tubes were incubated at 37° for 2 hours and allowed to stand overnight in the ice box before final reading was made.

Sera obtained at frequent intervals from patients acutely ill with pneumonia or convalescent from the disease have been mixed with varying dilutions of Fraction C and the presence or absence of precipitation noted. Charts 1 and 2 illustrate results which have been repeatedly obtained.

Chart 1 gives the results of observations in a case of Type II pneumococcus pneumonia. Serum obtained on admission to the hospital in the third day of disease reacted with high dilutions of Fraction C.

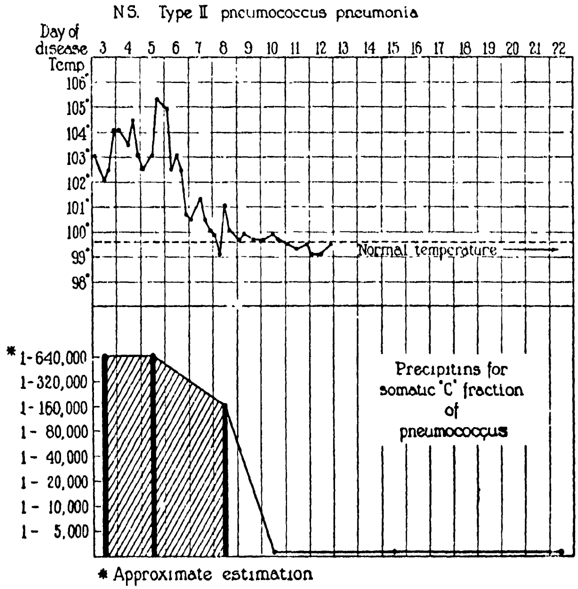

CHART 1

This reactive capacity dropped slightly on the day of crisis and completely disappeared in the next 2 days.

Chart 2 presents a similar course of events obtained with serum derived from a patient suffering from Group IV pneumococcus pneumonia. The phenomenon exhibited in Charts 1 and 2 has been observed in each of 50 patients ill with pneumococcus pneumonia. In each instance it has been found that serum obtained during the acute stage possesses a high titre of precipitins for Fraction C. A day or two after recovery this precipitating power is no longer detectable. The occurrence of the phenomenon is unrelated to the type of Pneumococcus causing infection. The fact that serum obtained from patients on admission possessed, in every instance, anti-C precipitins is evidence of the early appearance of the reactivity. The serum obtained from one patient as early as 18 hours after the initial chill reacted with high dilutions of C substance. Individuals who succumbed to the disease maintained C precipitins until death. The age of patients, whose sera have been tested, ranged from 7 years to 65 years; the phenomenon was present in the youngest and oldest. Sera from normal individuals have not been found to possess demonstrable precipitins for Fraction C.

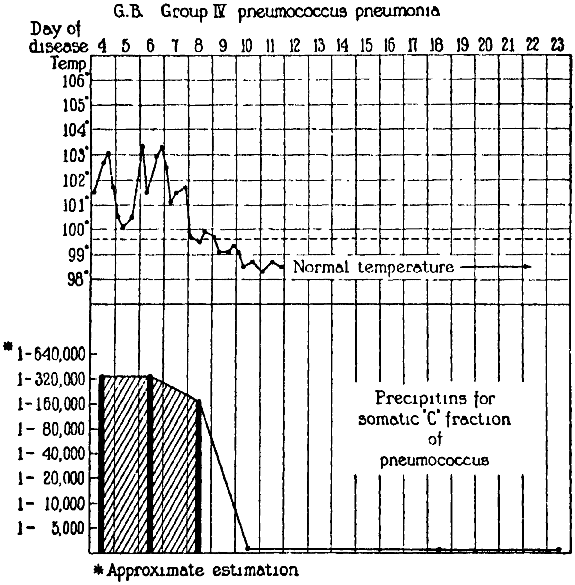

Chart 2

The observations on the serological reactivity of pneumococcus Fraction C presented in this report are limited to precipitin tests made with sera from patients. However, it may be mentioned that antipneumococcus sera derived from animals, in some instances, precipitate Fraction C. This is particularly demonstrable with the antipneumococcus horse sera[1] used for typing of pneumococci. The available lots of antisera of Types I, II and III all precipitate Fraction C, Type III serum apparently reacting in highest titre. Of sera derived from 24 rabbits immunized with either Types I, II, III, or R pneumococci, only 3 possessed demonstrable anti-C precipitins; each of these animals had received heat-killed cultures of Type III Pneumococcus. At the present time the conditions which favor the production experimentally of anti-C antibodies are not understood.

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[1] The antipneumococcus horse sera were obtained through the courtesy of Dr. A. B. Wadsworth from the New York State Board of Health Laboratories, Albany, New York.

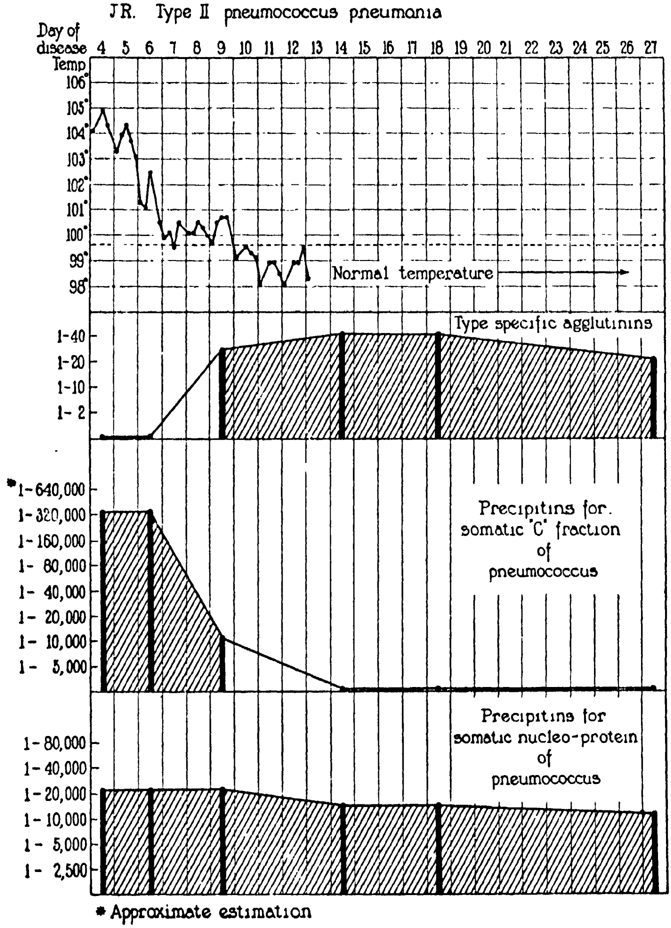

Chart 3

For purposes of comparison, sera derived from patients during the course of pneumonia were tested for the presence of type-specific antibodies and for pneumococcus protein antibodies as well as for C precipitins. The results when charted in relation to the course of pneumonia, present three different curves of antibody titre. Observations of this character are presented in Chart 3.

From Chart 3 it will be seen that type-specific antibodies are absent during the acute illness and are first demonstrable at the time of crisis, whereas precipitins for C substance are present in high titre during the febrile stage and are no longer detectable a few days after the critical fall in temperature. Antiprotein antibodies do not vary markedly during the course of illness, although there seems to be a slight reduction in titre as convalescence progresses. Observations similar to those given in Chart 3 have been made on twenty cases of pneumonia. It is an interesting fact, therefore, that with three chemically distinct fractions derived from the same bacterial cell, serological reactions involving three qualitatively different antibodies may be demonstrated during the progress of pneumococcus pneumonia.

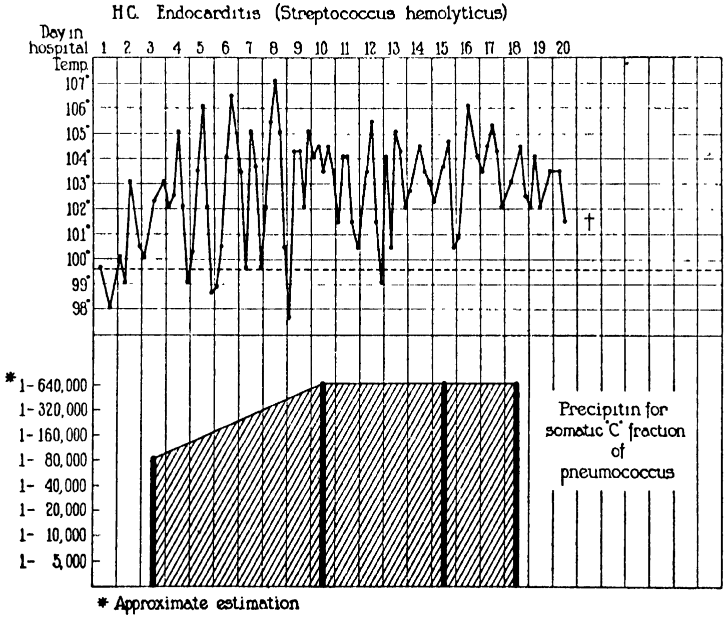

CHART 4

Up to this point the report has been limited to the results obtained with the sera of patients suffering from pneumococcus infection. A limited number of individuals acutely ill with other febrile diseases have been available for comparative tests. Four cases of hemolytic streptococcus infection ending fatally were observed from admission to the hospital until death. Three of these had streptococcus pneumonia. In each instance their sera precipitated pneumococcus Fraction C equally as well as did sera from cases of pneumococcus pneumonia. The fourth patient had malignant endocarditis; hemolytic streptococci in large numbers were repeatedly demonstrated in blood cultures. The result of serological tests made with pneumococcus C substance in this case is presented in Chart 4.

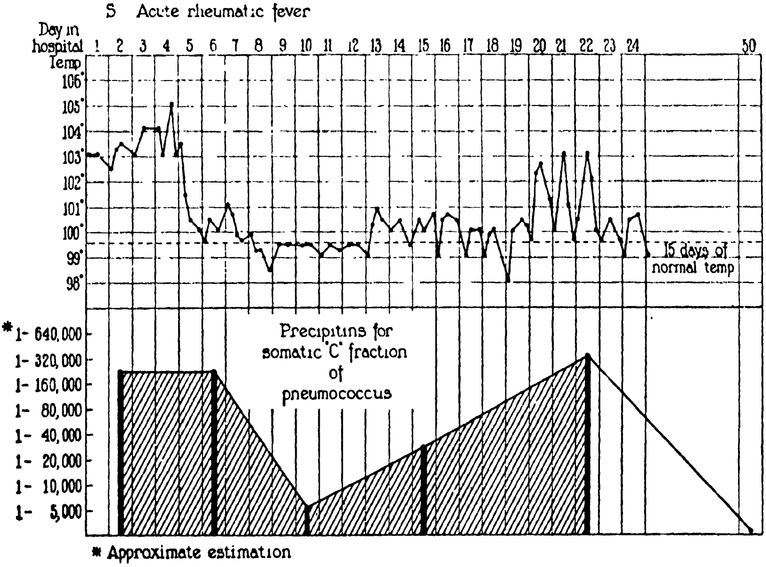

CHART 5

As can be seen from Chart 4 the patient's serum maintained throughout the illness the capacity to react in high titre with C substance of Pneumococcus.

Fifteen cases of acute rheumatic fever have been followed by similar serological tests through stages of active disease and remission. Chart 5 records the results obtained in one of this group.

As demonstrated by Chart 5 the course of the temperature and the capacity of the patient's serum to precipitate pneumococcus Fraction C closely parallel each other. The same relations were found to hold in the other fourteen cases of rheumatic fever. All of the patients at the time of elevated temperature exhibited symptoms and signs of active disease.

TABLE I

Reactions with Fraction C in Febrile Diseases Other than Pneumococcus Pneumonia

Patient	Disease	Temperature	*Dilutions of Fraction C		
			1-10,000	1-80,000	1-120,000
Das....................	Malaria	104°	–	–	–
Mek....................	Lung abscess	102.5°	+	++	+++
Fol....................	Cirrhosis of liver	103°	–	–	–
Mit....................	Typhoid fever?	104°	–	–	–
Rid....................	Pericarditis, rheumatic origin	103°	+	++	++
Reg....................	Unexplained anaemia	104°	–	–	–
Hag....................	Tuberculosis of lungs	102.5°	±	–	–
Mer....................	Unexplained fever—Tbc.?	103.5°	–	–	–
Hin....................	Osteomyelitis (staphylococcus)	105°	+++	+++	++
McD....................	Measles	102.8°	–	–	–
Pe....................	Chicken pox	103°	–	–	–
Mac....................	Endocarditis (*Streptococcus viridans*)	104°	+	+	±

* Approximate estimation.

In addition to the hemolytic streptococcus infections and cases of acute rheumatic fever just mentioned, single tests were made on sera obtained in a miscellaneous group of diseases. The results are listed in Table I.

From Table I it can be seen that patients, whose sera precipitated pneumococcus Fraction C, were in each instance suffering from disease associated with Gram positive cocci. The number of cases so far available is too few to attach undue significance to this point. However, the results suggest that etiological agent and serological reactivity with Fraction C may have some causal relationship.

DISCUSSION

The observations recorded in this communication have been made with a constituent of pneumococcus cells which is chemically distinct from both the type-specific capsular carbohydrate and the somatic nucleoprotein. Although pneumococcus Fraction C in all probability is a nitrogenous sugar, it differs from the soluble specific substance in that it possesses no type-specificity and is contained, as a common species constituent, within the body of the cells. Pneumococcus Fraction C appears to be analogous to the somatic carbohydrate isolated from hemolytic streptococci by Lancefield (10).

By testing sera obtained from cases of lobar pneumonia during the progress of the disease two interesting observations were made. The first of these is the early appearance of precipitins for C substance and the second is the complete disappearance of the phenomenon 1 to 3 days after crisis. Sera obtained from patients on the day of admission to the hospital have in every instance reacted with Fraction C. This was true even of cases admitted in the first 24 hours of disease. Since the serum of no normal individual has exhibited this reaction, the appearance of C precipitins is associated closely with the onset of pneumonia. Although an explanation of the phenomenon is not yet available, a possible interpretation is suggested by the so-called "anamnestic" reaction. Cole (13) found that in an animal which had previously been immunized and in which the antibodies in the blood had disappeared a subsequent injection of the original antigen caused antibodies to reappear more rapidly than did the primary injections. Bieling (14) employing two antigens (*B. dysenteriae* and *B. typhosus*), found that the reinjection of one brought back heterologous as well as homologous antibodies. In the course of similar experiments McKenzie and Frühbauer (15) using four antigens of remote biological origin were able to cause heterologous antibodies promptly to reappear by the injection of one antigen. Applying the principles involved in the "anamnestic" reaction to the observations recorded in this paper, it is possible that the occurrence of pneumococcus C precipitins early in pneumonia may be determined by a previous experience of the individual with some suitable bacterial antigen.

In addition to the observations with Fraction C, the investigation was extended to include a more complete study of the occurrence during pneumonia of antibodies reactive with pneumococcus constituents. In these latter experiments, therefore, tests were made for antibodies reactive with: 1. Type-specific capsular polysaccharide; 2. Non-type-specific somatic polysaccharide; 3. Non-type-specific somatic nucleoprotein. By charting the result of these serological reactions with reference to the course of the disease, the occurrence of each of the qualitatively different antibodies was shown to follow a different course. It is an interesting fact, therefore, that during a *single* infection, the body responds in such a manner that antibodies reactive with different components of the causative organism become demonstrable at different stages of the disease. The results serve to illustrate the complexity of the immunological mechanism which presides over the response to bacterial infection. The observations recorded in this paper also demonstrate that the reactivity of a patient's serum with pneumococcus C substance is not specific for pneumococcus infection. Sera from a limited number of febrile diseases due to other causes have been tested. In this small group, precipitation of C fraction occurred most definitely in those instances where Gram positive cocci were proven to be or were suspected of being the etiological agent. If the implications contained in these few cases are substantiated by a larger number of tests the results suggest some, as yet undetermined, broad relationship existing among certain bacterial infections.

SUMMARY

1. Sera from individuals acutely ill with lobar pneumonia possess the capacity to precipitate in high titre a non-protein somatic fraction derived from pneumococci (Fraction C). Following crisis the reaction is no longer demonstrable.

2. Sera obtained from cases of pneumococcus pneumonia during illness and convalescence have been tested for antibodies specifically reactive with three chemically distinct constituents of Pneumococcus. The results, when correlated with the course of disease, demonstrate differences in the occurrence of each qualitatively distinct antibody.

3. The precipitation of pneumococcus Fraction C is not limited to the sera of individuals ill with pneumococcus infection. But in the

few other cases available for comparative tests, definite reactions have been obtained only in streptococcus and staphylococcus infections and in acute rheumatic fever.

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CUTANEOUS REACTIONS IN PNEUMONIA. THE DEVELOPMENT OF ANTIBODIES FOLLOWING THE INTRADERMAL INJECTION OF TYPE-SPECIFIC POLYSACCHARIDE

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In a previous communication (1) the occurrence of cutaneous reactions to the polysaccharides and proteins of Pneumococcus during lobar pneumonia was reported. The investigation disclosed the interesting fact that the capsular carbohydrates of Pneumococcus were capable of inciting a reaction when injected intradermally. The character of the response and the conditions under which it was obtained are briefly summarized as follows: The characteristic reaction is the development of an *immediate wheal and erythema* at the site of injection; a positive reaction was first elicited at or about the time of crisis; it was produced only by the polysaccharide homologous in type to that of the Pneumococcus causing the disease; at the time of reaction type-specific antibodies for the homologous organism were demonstrable in the patient's serum.

In contrast to the polysaccharides, the so-called nucleoprotein fraction of the Pneumococcus evoked a *delayed* response which reaches its height in 24 hours. It simulated in appearance the tuberculin reaction. The protein reaction generally appeared first early in convalescence and tended to become progressively more marked during the course of recovery. Circulating antibodies for the nucleoprotein were present in approximately equal concentrations during the periods of acute illness and recovery. The protein reaction bore no specific relation to the type of Pneumococcus producing the infection.

The present report includes further observations on cutaneous reactions to pneumococcus fractions and deals especially with the appearance of antibodies for more than one specific type of Pneumococcus in the serum of convalescent patients. The individuals whose sera presented these unusual reactions had received repeated intradermal injections of the pneumococcus carbohydrates. The possible relationships between the repeated injection of the specific substances and the antibody formation will be discussed.

Materials and Methods

A. Skin Test Materials

1. Type-Specific Capsular Polysaccharides.—The type-specific polysaccharides were obtained in purified form from Types I, II and III Pneumococcus by the method of Heidelberger and Avery (2). They are chemically and serologically distinct and have been identified with the capsular material of the cell. The Type II and Type III polysaccharides are nitrogen-free; that of Type I contains 5 per cent nitrogen as part of the sugar molecule (3). The polysaccharides were diluted in physiological salt solution to a concentration of 1:10,000 and 0.1 cc. containing 0.01 mg. of the specific soluble substance was injected intracutaneously. Skin tests were repeated at intervals throughout the disease and convalescence.

2. Nucleoprotein.—The nucleoprotein was prepared by the method of Avery and Morgan (4) from an R strain derived from a Type II S organism. The material was standardized on the basis of nitrogen content and 0.01 mg. of protein in 0.1 cc. of physiological saline was used in all tests. These tests were repeated at approximately weekly intervals during the patient's stay in hospital.

B. Titration of Sera for Antibodies

At intervals during the acute illness and convalescence samples of serum were obtained and tested for the presence or absence of circulating antibodies.

1. Type-Specific Antibodies.—The presence in the serum of type-specific antibodies was determined by (a) agglutination, and (b) passive protection of mice.

a. Type-Specific Agglutinins.—0.5 cc. of varying dilutions of the serum to be tested was mixed with 0.5 cc. of a heat-killed suspension of type-specific pneumococci. Final readings were made after the mixtures had been incubated for 2 hours in the water bath and then placed in the ice box overnight.

b. Passive Protection of Mice.—The capacity of patient's serum to protect mice against infection was tested by the usual technique. 0.2 cc. of serum diluted to 0.5 cc. with salt solution was employed. So far as possible each serum was tested against pneumococci of Types I, II and III. The cultures used were invariably fatal for mice in doses of 0.0000001 cc.

2. Antibodies for Nucleoprotein.—Antibodies for the nucleoprotein material were determined by the precipitin reaction as previously described (1).

A study of the cutaneous response to the injections of the type-specific polysaccharides of the Pneumococcus and of the occurrence of circulating antibodies has been made in 74 cases of lobar pneumonia. The results confirm earlier observations and may be briefly summarized as follows: Positive reactions were elicited only with the homologous polysaccharide at crisis or shortly thereafter; homologous type-specific antibodies were demonstrable in the serum of each reactive patient. In no instance was a positive skin reaction to a polysaccharide obtained in the absence of circulating antibodies for the type of Pneumococcus from which the polysaccharide was derived. Table I shows the relative frequency of positive reactions to the homologous type of polysaccharide in patients convalescent from Type I, II or III pneumonia. All but 4 of the Type I cases were treated with anti-pneumococcus Type I serum.

TABLE 1

The Incidence of Cutaneous Reactions to the Homologous Polysaccharide in Convalescent Patients

Type of infecting organism	No. patients	No. positive reaction	Per cent giving positive reactions
I	21	21	100
II	17	10	58.8
III	9	4	44.4

13 recovered cases of Group IV—no reactions to polysaccharides of Types I, II, III.

5 recovered cases of Type II (atypical)—no reactions to polysaccharides of Types I, II, III.

9 fatal cases of various types—no reactions to polysaccharides of Types I, II, III.

From Table I it can be seen that positive cutaneous reactions were obtained in all of the Type I cases and in approximately 50 per cent of the Types II and III patients. The polysaccharides of Types I, II and III elicited no response in the 13 Group IV and the 5 atypical Type II patients. The absence of reactivity in the 9 fatal cases agrees with earlier experiences in that in patients with lethal termination no reaction was obtained. 12 patients convalescent from Type II or Type III pneumonia also failed to react but in 8 of them no reaction would be expected since their sera contained no circulating antibodies; the sera of the remaining 4 patients contained circulating antibodies but no reaction to the polysaccharide occurred. Thus, of the entire group of patients included in Table I a positive skin reaction was expected in 39 by virtue of the fact that they were recovered cases of Type I, II or III infection whose sera contained type-specific antibodies. Positive reactions were obtained in 35 of them.

The cutaneous response to the nucleoprotein fraction of the Pneumococcus was studied in the same group of cases. The results have confirmed the validity of the earlier conclusions. The delayed protein reaction appeared first during convalescence. The reactivity of the patient did not appear to be influenced by the titer of circulating antibodies for the protein fraction.

Occurrence of Antibodies to Heterologous Types of Pneumococcus

Other observers have repeatedly demonstrated the occurrence of circulating antibodies for the homologous type of Pneumococcus at or about the time of crisis. The present investigation confirms these results and, in addition, discloses the fact that at the time when type-specific antibodies are first demonstrable in the blood the patient becomes reactive to the cutaneous injection of the homologous type-specific polysaccharide. The reactions have been specifically limited to the homologous types. In the present study repeated tests were made with the type-specific polysaccharides of Types I, II and III with a view to determining the length of time through which the reactive capacity might persist. In view of the fact that positive results at the time of recovery were strictly limited to the homologous type, it was rather surprising to observe after repeated tests that a positive skin reaction was obtained in certain instances with a polysaccharide of heterologous type as well. With the appearance of the skin reaction to the heterologous polysaccharide, circulating antibodies for the heterologous type of Pneumococcus were demonstrable in the patient's serum, in addition to those for the homologous type. The chief interest in this report, therefore, is concerned with the development of specific antibodies for more than one type of Pneumococcus in patients who have repeatedly received intradermal injections of type-specific carbohydrates. These "additional" antibodies were characterized by the fact that they were heterologous in type to that of the infecting organism and were not demonstrable at crisis but appeared 1 to 3 weeks after the patient's recovery. These features were disclosed as a result of what at first appeared to be a discrepancy in specificity of the cutaneous reaction. The results can be more clearly brought out by describing in detail the course of events in one patient.

M. A., a girl of 14 years, suffering from Type II pneumonia was admitted on the second day of disease. No circulating antibodies were demonstrable in the patient's serum at this time. Crisis occurred on the tenth day. On the thirteenth day skin tests with the polysaccharides of Types I, II and III revealed a positive reaction only to the Type II material. The serum taken shortly thereafter contained circulating antibodies for Type II Pneumococcus alone. 8 days after the first test the skin tests were repeated and at this time positive reactions occurred both to Type II and Type I polysaccharides. At this time her serum contained antibodies for Type I as well as for Type II Pneumococcus. (See Chart 1.)

Because of the appearance of heterologous antibodies and skin reactions a series of patients was studied in order to determine the frequency with which the phenomenon occurred. Skin tests and antibody determinations were made at varying intervals in 18 patients. Of these, 3 were cases of Type I pneumonia, 7 of Type II, 3 of Type III and 5 of Group IV. At or about the time of crisis antibodies for the homologous type of Pneumococcus appeared in all but 2 of the Types I, II and III patients. In the Group IV cases tests were not carried out with the homologous organism. However, in no instance were heterologous antibodies demonstrable at that time. As convalescence progressed, repetition of skin tests and antibody determinations revealed that there had *developed,* since they were previously absent, specific antibodies and a positive skin reaction for at least one of the heterologous types of Pneumococcus. Table II shows the intervals between the appearance of homologous and heterologous antibodies in relation to the day of disease and recovery.

From Table II it can be seen that in 10 of the 18 patients heterologous antibodies were detected during convalescence; in 3 Type I patients, 4 Type II, 2 Type III, and 1 of Group IV. In the great majority of cases the presence of circulating antibodies for the heterologous type was first indicated by a positive skin test to the polysac-

TABLE II

Differences in Time of Appearance of Homologous and Heterologous Antibodies in Patients Receiving Skin Tests with Type-Specific Polysaccharides

Patient	Antibodies to homologous type		Antibodies to heterologous types				Day of crisis
	Type	Day observed	Type			Day observed	
L. H. (serum), No. 7165	I	3			III	17	6
M. P. (serum), No. 7263	I	8		II		14	5
F. R., No. 7253	I	9		II		22	7
M. C., No. 7183	II	14	I			19	5
G. M., No. 7248	II	10	I			19	9
E. C., No. 7269	II	11	I			18	9
M. A., No. 7261	II	15	I			22	10
H. R., No. 7262	III	6		II		19	
			I			26	8
K. R., No. 7143	III	5	I	II		21	4
A. P., No. 7211	IV	Not determined	I			26	
					III	44	7
M. C., No. 7189	II	15	0	0	0		8
I. S., No. 7195	II	9	0	0	0		6
C. Q., No. 7225	II	0	0	0	0		7
J. F., No. 7171	III	0	0	0	0		10
G. B., No. 7178	IV	Not determined	0	0	0		8
R. F., No. 7256							9
T. R., No. 7197							5
G. R., No. 7236							8

charide of that type. The type of Pneumococcus for which heterologous antibodies developed was in 7 instances Type I, in 4 Type II, and in 2 Type III. In 3 cases specific antibodies for 2 heterologous types appeared. Although the homologous antibodies were detectable at the time of crisis, those for heterologous types were usually noted in the second week of convalescence, the earliest being 9 days after recovery, the latest 37 days after recovery. The period of time during which antibodies were detectable in the serum and skin reactivity remained positive varied from a few days to several months.

These observations led to the hypothesis that the mechanism involved in the production of antibodies for heterologous types of Pneumococcus was associated with the intradermal inoculations of the type-specific polysaccharides. In order to obtain more information on this point the next 21 pneumonia patients admitted to hospital were studied as controls. In this series no injections of the specific polysaccharides were made but the serum was tested at intervals for circulating type-specific antibodies to Pneumococcus Types I, II and III. In the Group IV cases tests were not made for antibodies reactive with the infecting organism. In most of the 21 cases homologous antibodies were observed at the time of recovery. However, in striking contrast to the group of patients which had received repeated injections of the specific soluble substance, no patient in the control series *developed* antibodies for a heterologous type. In only 3 patients (1 each of Types I, II and Group IV) antibodies for a type other than that of the infectious agent were observed. In these three instances the presence

TABLE III

The Incidence of Type-Specific Antibodies in the Control Series of Patients

Type of infecting organism	No. cases	Antibodies for homologous type of Pneumococcus	Antibodies for heterologous types of Pneumococcus
I	6	5	1*
II	6	6	1*
III	2	2	0
Group IV	7	Not determined	1**

* Type III antibodies present during disease and recovery.

** Type II antibodies present during disease and recovery.

of the heterologous antibodies was detected in low titre early in the acute illness and the concentration of the antibodies did not increase during convalescence. They, therefore, bear no apparent relation to the present infection but are presumably attributable to a preexisting state. This interpretation is substantiated by the fact that some normal individuals possess in their serum type-specific antibodies.

Table III presents the distribution of the types of Pneumococcus in the control group of patients and the number in whom circulating antibodies were detectable.

The 21 patients who served as controls were comparable to the test group with regard to age incidence, distribution of types of infecting Pneumococcus, severity of the infection and period of observation.

The patients in whom heterologous antibodies were observed, received injections of 0.01 mg. of the type-specific capsular polysaccharides derived from the Pneumococcus of Types I, II and III. The average number of injections before the appearance of antibodies for the corresponding type of Pneumococcus was three. In 3 cases the antibodies were detected after a single injection. The largest total amount which was followed by heterologous antibody production was 0.05 mg. whereas in other cases heterologous antibodies did not develop even after the injection of as much as 0.08 mg. of the polysaccharides. These irregularities demonstrate the fact that the production of antibodies is not solely dependent upon the quantity of polysaccharides injected. The average time between the last injection and the appearance of the antibodies was 6 to 10 days but in one case they were first detected 20 days after the last injection. The exact data regarding the individual patients in whom antibodies for heterologous types of Pneumococcus developed are presented in graphic form in composite charts. These charts show in relation to the day of disease, the time of appearance of type-specific agglutinins and mouse protective antibodies for homologous and heterologous types of Pneumococcus, the time, number and results of skin tests with the 3 type-specific polysaccharides and the period during which the observations were continued.

DISCUSSION

The primary interest of the present work centers in a group of 18 cases of lobar pneumonia 10 of whom, in convalescence, developed

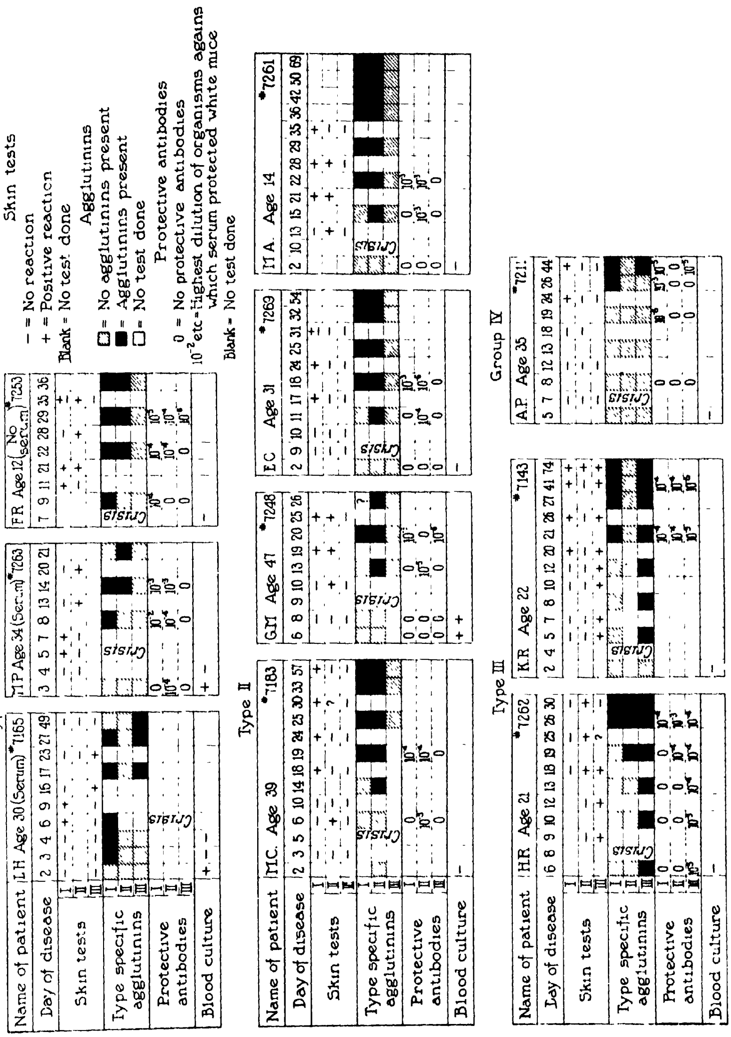

CHART 1. The development of heterologous antibodies in patients receiving intradermal injections of type-specific polysaccharides.

circulating antibodies for at least one type of Pneumococcus heterologous to that etiologically related to the disease. All of the patients in whom the phenomenon was observed had received repeated intradermal injections of 0.01 mg. of specific capsular polysaccharide from each of Types I, II and III Pneumococcus. Antibodies for the homologous type of Pneumococcus appeared at about the time of crisis. Antibodies for heterologous types, on the other hand, appeared usually in the second week of convalescence after 1 to 5 injections of the respective polysaccharides.

A control group of patients, 21 in number, received no skin tests. Their sera, in the great majority of instances, contained antibodies for the homologous type of Pneumococcus. In 3 of these cases antibodies for a heterologous type were present but the time of appearance differed from that of the antibodies observed in the first group in that they were detectable in low concentration not only in convalescence but during the acute illness as well. Nor was any increase in concentration observed during convalescence. Antibodies to the heterologous types which were encountered in these 3 cases, apparently preexisted and their appearance is presumably unrelated to any incident occurring in the course of the present infection. The validity of this supposition is borne out by the fact that type-specific antibodies may be found in the serum of certain normal individuals, who give no history of lobar pneumonia. But, in no case were heterologous antibodies first detected in convalescence unless the patient had previously received intradermal injections of the type-specific polysaccharides.

Many investigators have noted the presence of specific antibodies for the homologous strain or type of Pneumococcus in the blood of pneumonia patients at the time of crisis. Bacteriotropins (5, 6, 7), agglutinins (5, 7, 8, 9, 10, 11, 12) and protective antibodies (13, 14, 15, 6, 12) have all been demonstrated but so far as can be discovered, in no instance has the development of antibodies for a heterologous type been described. Chickering (11) in 1914 followed the course of agglutinin production to all types throughout convalescence in 40 cases but found only antibodies for the homologous type. Clough (7) in 1919, using the serum of patients convalescent from pneumonia due to known types of Pneumococcus, obtained similar results with phagocytic and agglutination reactions. In the sera of 25 normal individuals, however, Clough (16) found protective antibodies for Type I in 4 of 18 tests, for Type II in 8 of 18 tests; and of 19 tests for Type III antibodies, 8 were positive. Ward (20) tested the capacity of the defibrinated blood of normal individuals to inhibit the growth of pneumococci of Types I, II and III. In each instance a variable degree of pneumococcidal action was observed against one or more types of the organism. Furthermore, this property was present in the blood of patients early in the course of pneumonia even against the type of Pneumococcus producing the infection. Robertson and Cornwell (21), employing serum-leucocyte mixtures from normal human beings, found that the blood of all persons tested by this method possessed pneumococcidal properties for at least one type of Pneumococcus and in the majority of instances for two or more types.

In evaluating the factors involved in the production of antibodies for types of Pneumococcus heterologous to that causing the infection several possible mechanisms may be considered.

It is possible that antibodies for other types were present before the onset of disease and were masked during the acute infection only to return with convalescence. Or that, present in minimal amounts before the disease, the infection served to cause an outpouring of antibodies in detectable concentrations,—the anamnestic reaction first observed by Cole (17).

Interconvertibility of types as described by Griffith (18) and later studied by Dawson (19) offers a possible explanation. One might assume that with the development of type-specific antibodies to the homologous organism a change of type occurred and, as a result, antibodies to the secondary type appeared.

The evidence presented in this report implicates the polysaccharides employed in intracutaneous tests and suggests that they were antigenic. On the basis of this assumption, the conditions under which the investigation was carried out may have favored antibody production. No previous data are available regarding the antigenicity in humans of the purified type-specific polysaccharides of the Pneumococcus. On the basis of animal experiments in this laboratory these substances, in the pure state, are considered non-antigenic. It is possible, however, that in the process of recovery from infection, a highly reactive state exists in the human organism which responds to stimuli otherwise ineffective. In the removal of the inflammatory material from its focus in the lungs, as takes place in pneumonic resolution, the absorbed material may act as an adjunct to the polysaccharides (the "Schleppe" of the German investigators) forming a combination possessing antigenic properties. The polysaccharides which, under ordinary conditions, are haptens, may then acquire antigenic properties and determine the specificity of the antibodies produced. It is of course also possible that the particular preparations used in this study, although they were prepared with great care, still contained minute amounts of combined polysaccharides.

At present it is impossible to offer any final statement as to the exact mechanism by which antibodies for the heterologous types are produced. Further work is being carried on with normal individuals and patients in an attempt more completely to elucidate the factors which play a rôle in the development of these heterologous antibodies.

SUMMARY

The majority of patients convalescent from pneumonia due to Types I, II and III Pneumococcus develop at the time of recovery circulating antibodies for the homologous type of organisms. At the same time an immediate wheal and erythema reaction followed the intradermal injection of the homologous type-specific polysaccharide in 100 per cent of Type I patients, 58.8 per cent of Type II patients, and 44 per cent of Type III patients.

In a group of 18 patients repeatedly tested with the type-specific polysaccharides, 10 developed in the second or third week of convalescence circulating antibodies for one or more heterologous types. In none of 21 control patients was this phenomenon observed.

It is suggested that the development of circulating antibodies for heterologous types of Pneumococcus was associated with the previous intradermal injections of the type-specific polysaccharides.

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ANTISTREPTOCOCCUS SERUM TREATMENT OF PATIENTS WITH RHEUMATIC FEVER

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In the present stage of the therapy of rheumatic fever, any promising remedial agent deserves serious consideration. Except for the antiarthritic and antipyretic drugs, together with the usual measures of strict hospital routine, we have been without means of influencing the course of this infection. It is true that in certain instances these drugs have been reported to exert a favorable effect upon prolonged conduction time,[1] and that occasionally the symptoms of carditis respond in as pleasing a fashion as does arthritis; nevertheless common experience has taught that the salicylate effect usually represents an interlude in the course of a disease which, once established, frequently proceeds relentlessly to the production of serious and often eventually fatal lesions. The appearance of subcutaneous nodules in patients under the full influence of salicylate or neocinchophen has been cited as demonstrating the inability of these drugs to prevent the occurrence of more deeply seated damage concomitantly wrought. The importance of the claims of Small,[2] that the serum developed by him is competent to arrest this damage, can, therefore, scarcely be overestimated; and in view of his results, as well as of the enthusiastic report of Baruffaldi,[3] together with the claims of earlier investigators, it has seemed well worth while to investigate further the effects of antistreptococcus serum upon the course of the disease.

In view of the possibility that many types of streptococci may act in the causation of the disease in subjects peculiarly hypersensitive, it seemed reasonable to attempt to determine whether different antistreptococcus sera might not have similar therapeutic effects.

Commercial antitoxic and antibacterial antiscarlatinal serum prepared by the New York City or State Health Departments was employed as antihemolytic streptococcus serum. Through the kindness of Dr. Small, a quantity of S.C.A. (indifferent streptococcus) serum, both bovine and equine, was made available. The equine serum was in concentrated form. For the production of anti-green-streptococcus serum a strain, V110A, originally isolated from an excised subcutaneous nodule, was chosen. With this organism a heifer was immunized by the intravenous route, first with heat-killed vaccine, eventually with large quantities of living culture. After several months' immunization the animal was bled, and about 3 liters of serum of agglutinin titer approximately 1 to 5000 were obtained. This serum, hereinafter referred to as V110A bovine, was stored in the icebox without preservative.

The intramuscular route of administration was always employed, except in one instance when the serum was given intravenously. Suitable intradermal tests with normal sera were always carried out, and, except in two cases referred to below, no serum to which there was evidence of skin sensitiveness was employed.

The evaluation of the effect upon rheumatic fever of any therapeutic measure is a matter of no mean difficulty. The universal application of salicylate therapy has served in a measure to obscure the really tremendous variability in the course of the disease, particularly with respect to such aspects as fever and arthritis. Reference to the older literature[4] reveals how well this variability was realized before the introduction of antirheumatic drugs. It has been no unusual experience with us to observe marked improvement in these manifestations, occasionally even complete relief, under expectant therapy alone, within a few days of hospitalization. In the absence of adequately prolonged control periods, such improvement might well have been ascribed to serum therapy, had this been undertaken shortly after the admission of the patient. With regard to severe carditis or to pericarditis, the known tendency of these conditions to pursue more or less self-limited cycles has removed uncertainty to some extent, provided that the treatment has been undertaken sufficiently early in the cycle to warrant the supposition that spontaneous improvement has not been at hand. Even so, however, the uneven course of the disease forbids too dogmatic an interpretation of results.

In this study, emphasis has been laid upon an attempt to secure adequacy of the control period, save only when the condition of the patient has rendered immediate action imperative. In the case of arthritic patients, it seemed desirable to give serum only after several days' observation had apparently established that the disease was running an irregularly continuous course without remissions, or at best with slight and temporary ones; or when, in spite or irregularities in temperature, intensification of the arthritis, with involvement of fresh joints, seemed to indicate the imminence of renewed activity of the infection. Even so, it is with a certain degree of reservation that the occasional improvement of joint symptoms has been attributed to the serum. As regards carditis and pericarditis, the indications have been much clearer; lengthy control periods have usually been precluded, because in each instance the patient has been in the grip of so serious an infection that rapid spontaneous recovery has seemed out of the question. Favorable results observed in this group we have felt might be ascribed largely to the therapy employed, which, let it be noted, was a combination of serum and drug.

The relief only of the major manifestations of rheumatic fever cannot be taken to indicate recovery from the disease, in view of the chronicity of manifestations which may be masked by the use of salicylates. Such items as persisting leukocytosis, subfebrile temperature, failure to gain weight, and variability of the electrocardiogram are now recognized as indicative of persistent infection. No less important, therefore, than adequacy of the preliminary control is minute observation of the patient during the period through which the serum effect may reasonably be expected to persist. This is placed by Small at three weeks or more. It is of course of paramount importance for a proper study that drugs should be withheld during this period. In our study the period of withholding other remedies has only occasionally be so extended, either because of absence of beneficial effects within a few days or because of relapses well before the termination of the prescribed interval. Such relapses, observed not later than two weeks following the treatment, may reasonably be considered as therapeutic failures regardless of the degree of temporary benefit observed. After all, temporary benefit may usually be rapidly secured with antirheumatic drugs; hence before these can be supplanted by serum, it must be shown that the action of the latter is comparably efficient and reasonably prolonged. With these considerations in mind, we have endeavored to determine as accurately as possible the post-therapeutic course of our patients. The results of the observations have been summarized upon special charts as well as in bedside notes.

To judge critically a single therapeutic agent one should eliminate other favorable influences which arise from hospitalization, adequate nursing, complete rest and proper food; yet these measures are requisite for the patient's welfare. It seems scarcely necessary to emphasize how much more confusion would result if, simultaneously with the serum therapy, were exhibited such drugs as sodium salicylate in arthritis, or bromids or luminal in chorea. Such a study might illustrate still further the efficacy of the drugs, but would be without value with respect to the serum. As far as possible, therefore, we have, except in the case of severely ill patients, abstained from the use of salicylates until satisfied of the failure of the serum. Unfortunately, in a few instances, salicylate was administered at or before the time when the serum effect might have been manifest, and thus the post-serum control period has been inadequate. These few patients, observed in a preliminary study, are included only for the sake of completeness. In the presence of circulatory failure, it has similarly seemed preferable to withhold digitalis insofar as possible. While it is theoretically improbable that this drug would influence an active rheumatic infection, the antipyresis occasionally observed following its administration to patients with congestive failure might confuse the picture were it employed in combination with serum therapy.

Occasionally other irregularities have crept in, as stated in the footnotes to the tables. Attempts at desensitization of two serum allergic patients did not prevent alarming anaphylactoid manifestations after larger doses of serum. Sometimes intradermal tests have been carried out with streptococcal proteins, either during the period of treatment, or shortly before. Although the combined dosage of these proteins never exceeded 0.03 mg., we have subsequently come to realize that this amount is at times sufficient to determine general and focal exacerbations of the disease.

The results are summarized in Tables I to IV, and in illustrative case histories at the end of the paper. In accordance with the advice of Dr. Small, the large doses originally employed were later replaced by smaller ones; consequently it has been necessary to subdivide the tables to follow the alteration of method.

Comment upon these results is interesting. The one patient with chorea to whom the serum was given failed entirely to show evidence of improvement. A disappointing failure to influence polyarthritis favorably has been encountered, for among 12 patients with this symptom only 3 showed complete and permanent relief; 2 after S.C.A. serum and 1 after antiscarlatinal. Various degrees of improvement which because of their evanescence can only be classed as failures, were noted following all three sera. Surely this result is eloquent. Were salicylate as ineffectual as serum in this condition, its use would long since have been abandoned. Four patients with carditis showed no evidence of even temporary improvement; in 2 of them the disease progressed steadily to death. In Case 4 a severe carditis developed well within a week of the completion of the serum therapy, and shortly thereafter a crop of subcutaneous nodules appeared. When such nodules were present before the institution of serum therapy, their normal involution showed no acceleration. In the case of pericarditis, however, an attitude of complete pessimism seems unwarranted. It is possible that the combination of drug therapy, given because of the serious character of the symptoms, together with the serum was responsible for the favorable results. Such mixed treatment doubtless confuses validity of conclusions, though it may be argued that drugs alone usually exert only a mild influence upon the course of pericarditis. But the prompt and progressive improvement following the combined therapy in Cases 7, 11 and 17 was probably not attributable entirely to the drug. Although the effect of spontaneous improvement might possibly be evoked in Cases 7 and 11, it is unlikely it would apply in Case 17, where treatment was given at the upturn of the cycle. Obviously, results from such small series as the present cannot settle the point, but can merely indicate possibilities which can be affirmed only after extensive and painstaking observation.

Various reactions quite apart from the usual manifestations of

TABLE I

Results with S. C. A. Serum

Case No.	Name.	Age.	Type of disease.	Day.	Length of control period, days.	Dose (A) in cc.	Reactions (B).	Therapeutic effects.	Remarks.
1*	M. S.	39	Arthritis, carditis, pulmonary	30	1	60 (3) Equ.	Moderate temperature and pulse (1, 2, 3)—5 to 10 hrs.; moderate arthritic (1, 2)—24 hrs.; râles increased (1)—24 hrs.; new murmur (1)—24 hrs.	None	Neocinchophen given after 48 hrs.; prompt relief and progressive improvement.
2**	G. H. (C. R.)	7½	Carditis, circutory failure	7	6	53 (2) Equ.	None	Slight antipyretic; pulmonary signs appeared on day of second treatment	Became steadily worse; required digitalis; neocinchophen given 2 days after completion of treatment, with moderate improvement only; 2 days later large pleural effusion.
3	J. I.	15	Carditis, nodules, circulatory failure	4th yr.	157	20 (3) Equ.	Shock and chill (1)—30 min.; marked temperature and pulse (1)—8 hrs.	None	Eventually died.

4	S. E. (C. R.)	6	Arthritis, carditis	6	3	5 (2) Equ.	Moderate arthritic (1)—24 hrs.; slight temperature and pulse (2)—3 hrs.; marked respiration (2)—7 hrs.; gallop accentuated (1)—24 hrs.	Marked antiarthritic; otherwise none	Four days later relapse with arthritis and carditis; 1 week later nodules.
5†	A. R.	16	Arthritis, carditis	15	3	10 (3) Equ.	Moderate temperature (1)—7 hrs.; severe temperature; moderate pulse and respiration (2, 3)—6 to 7 hrs.; stupor and delirium (1)—7 hrs.; lasted 3 days; ectopic beats (1, 2)—24 hrs.	Marked antiarthritic; marked antipyretic; marked antitoxic and marked anticarditic	During treatment received large doses of neocinchophen; all improvement could be ascribed to this; severe relapse on drug withdrawal 3 weeks later.
6	A. E.	25	Arthritis, pulmonary	34	5	10 (3) Equ.	Moderate temperature (1, 2, 3)—5 to 11 hrs.; marked pulse and respiration (1)—9 hrs.; new pulmonary signs (1)—24 hrs.	Moderate antiarthritic; slight antipyretic; pulmonary condition unaffected.	One week later temperature became higher, pulmonary condition worse and relapse of arthritis; responded promptly to neocinchophen.
7††	R. M. (C. R.)	18	Arthritis, pericarditis	22	9	10 (3) Equ.	Moderate temperature and pulse (2)—6 to 10 hrs.; slight arthritic (2)—24 hrs.	Marked antipyretic; marked antiarthritic; marked antipericarditic	Severely ill; neocinchophen given for 1 week before treatment without effect and continued in small doses during treatment; pleuritic relapse 2 weeks after treatment; thereafter did well.

TABLE I—*Concluded*

Case No.	Name.	Age.	Type of disease.	Day.	Length of control period, days	Dose (A) in cc.	Reactions (B).	Therapeutic effects.	Remarks.
8	M. Z.	41	Arthritis	19	6	24 (2) Bov.	Slight arthritic (1)—24 hrs.	Antiarthritic, almost complete; antipyretic complete	Permanent; no drug necessary.
9	C. K. (C. R.)	30	Arthritis	44	5	50 (3) Bov.	Slight temperature (2, 3) —4 to 8 hrs.	Moderate, antiarthritic and moderate antipyretic for 24 hrs.	Thereafter severe relapse; neocinchophen given 9 days after completion of treatment; prompt relief.
10	F. P. (C. R.)	33	Arthritis, carditis	17	2	40 (2) Bov.	None	Marked antipyretic; marked antiarthritic; marked anticarditic	After 3 days a pericardial rub appeared for 48 hrs.; no drug necessary.
11‡	M. F. (C. R.)	22	Pericarditis, carditis, arthritis	93	9	50 (3) Bov.	Moderate temperature (1, 2)—3 to 4 hrs.; marked pulse and respiration (1)—3 hrs.; delirium, toxemia, cyanosis (1)—3 hrs.	Marked antipyretic; marked anticarditic; marked antipericarditic; marked antitoxic	Neocinchophen begun on first day of treatment on account of serious condition; no relapse followed drug withdrawal at end of serum course.

12‡‡	J. G.	10	Pericarditis, arthritis	5	1	19 (3) Bov.	Moderate arthritic (1, 2) —24 hrs.	Moderate antipyretic; slight antiarthritic; heart worse	Neocinchophen given 36 hrs. after completion of treatment; prompt but temporary drug effect, with pericarditic and pleuritic relapse after 5 days.
13§	S. R.	7½	Chorea	41	37	27½ (2) Bov.	Slight temperature (1)—8 hrs.; slight pulse (1)—12 hrs.	None	Given 0.01 mg. streptococcus protein i. v. 2 days before beginning treatment.

(A) Figures in parentheses indicate days over which total dosage given was distributed.

(B) Figures in parentheses indicate dose after which reaction occurred. Periods of time following dashes indicate interval elapsing before reaction was noted.

* Insufficient control and observation periods; included only for sake of completeness. Skin tests with 0.03 mg. streptococcus proteins on day following completion of treatment. Not included in Table IV.

** Observation period of only two days because of rapid deterioration.

† Not included in Table IV.

†† Treatment begun on eleventh day of pericarditis.

‡ Admitted with arthritis only; developed pericarditis under observation; treatment begun on fifth day of pericarditis.

‡‡ Insufficient control and observation periods; included only for completeness. Skin tests with 0.03 mg. streptococcus proteins on first day of treatment. Not included in Tables IV, V and VI.

§ Treatment given during a course of immunization with hemolytic streptococcus protein.

C. R.—For case reports see end of paper.

TABLE II

Results with V110A Serum

Case No.	Name.	Age.	Type of disease.	Day.	Length of control period, days.	Dose (A) in cc.	Reactions (B).	Therapeutic effects.	Remarks.
14	M. B.	22	Arthritis	7	3	40 (2)	Slight arthritic (1)—24 hrs.	Slight antiarthritic and slight antipyretic, 20 hrs. after first dose.	Thereafter much worse.
15	H. C.	19	Carditis, arthritis, nodules	35	8	40 (2)	Moderate temperature (1, 2)—4 to 8 hrs.; cyanosis and restlessness (1)—12 hrs.	Slight antiarthritic for 4 days; otherwise none	Became progressively worse, fibrillated after 2 weeks; neocinchophen given after 5 days.
16*	J. S.	48	Arthritis	30	1	45 (2)	Moderate temperature and pulse (1)—4 to 8 hrs.; dyspnea and cyanosis (1)—4 hrs.	Antiarthritic, almost complete, for 6 days	No antipyretic effect; after 6 days severe relapse, controlled only by full doses of neocinchophen.
17†	A. P. (C. R.)	8	Pericarditis, pulmonary	18	11	20 (2)	None	Marked antipyretic; cardiac and pulmonary condition improved gradually	Received neocinchophen 2 gm. daily during treatment; 1 week later rub returned for 3 days, then settled down into subacute course.
18	R. F.	33	Arthritis	43	5	47 (3)	Marked arthritic (1, 2, 3)—24 hours.	Worse	Three days later given neocinchophen with prompt improvement.

19	W. F. (C. R.)	41	Arthritis	26	2	40 (2)	Moderate temperature and pulse (1, 2)—3 to 6 hrs.; slight arthritic (1, 2)—24 hrs.	Marked antipyretic; marked antiarthritic	After four days severe febrile and arthritic relapse, yielding promptly to neocinchophen.
20	I. S.	18	Arthritis, carditis	78	34	50 (3)	Moderate pulse (3)—2½ hrs.	None	After 3 days entered upon a moderately severe cycle of fever and arthritis.

(A) Figures in parentheses indicate days over which total dosage given was distributed.

(B) Figures in parentheses indicate dose after which reaction occurred. Periods of time following dashes indicate interval elapsing before reaction was noted.

* Skin tests with streptococcus proteins on first day of treatment

† Neocinchophen 2 gm. q.d. during treatment.

C. R.—For case report see end of paper.

TABLE III

Results with Scarlatinal Antitoxin

Case No.	Name.	Age.	Type of disease.	Day.	Length of control period, days.	Dose (A) in cc.	Reactions (B).	Therapeutic effects.	Remarks.
21*	L. B.	48	Arthritis, pulmonary	19	5	55 (3)	Slight temperature (1, 3)—4 to 5 hrs.; moderate pulse (1, 2)—4 to 5 hrs.; moderate arthritic (1, 2, 3)—24 hrs.; râles increased (1)—24 hrs.	Slight antiarthritic	Disease apparently on the upgrade when sodium salicylate was given 2 days later.
22†	B. S.	19	Arthritis	23	3	23 (1)	Moderate temperature, 8 hrs.; rash, 24 hrs.; local, 8 hrs.	None at once; after 2 days fever and new murmurs; after a further 4 days pericarditis with effusion.	
23‡	A. S.	30	Arthritis	15	2	16 (2)	Marked dyspnea and cyanosis (2)—5 hrs.; slight temperature and pulse (2)—8 hrs.; marked arthritic (2)—24 hrs.	Slight antipyretic only	Because of poor condition neocinchophen given after 2 days; prompt relief.
24	R. McM.	18	Arthritis	70	8	6 (2)	None	Marked antipyretic; marked antiarthritic	Incomplete recovery; neocinchophen started after fifth day; relapse after 17 days.

25	N. S. (C. R.)	45	Arthritis	27	11	10 (4)	Marked arthritic (1)—24 hrs.	Marked antiarthritic, marked antipyretic	Three days later mild relapse of 6 days' duration; thereafter permanent relief.
26§	P. McD.	13	Carditis, arthritis, pulmonary	3	2	10 (3)	Vomiting (1) several hours	Moderate antipyretic, moderate antiarthritic after first dose only; cardiac and pulmonary condition worse	After 2 days given neocinchophen with prompt antipyretic and antiarthritic effect; other conditions improved more slowly but steadily.

(A) Figures in parentheses indicate days over which total dosage given was distributed.

(B) Figures in parentheses indicate dose after which reaction occurred. Periods of time following dashes indicate interval elapsing before reaction was noted.

* Observation period of only forty-eight hours robs case of value. Not included in Table IV.

† Attempt to desensitize a sensitive patient, with unpleasant sequellæ. Not included in Tables IV, V and VI.

‡ Desensitization unsuccessful; very severe serum shock. Observation period forty-eight hours only, on account of poor condition of patient. Not included in Tables IV, V and VI.

§ Received *unconcentrated* serum. Only forty-eight hours' observation period because rapidly becoming worse.

C. R.—For case report see end of paper.

serum sickness have been noted, irrespective of the type of serum employed. The most usual occurrence has been a rise in the temperature, occasionally quite abrupt, at an interval of from three to eight hours following injection. The interpretation of the fever curves is not always an easy matter, because of the innate unevenness of their course; in questionable cases one must judge as well as possible by comparison with the control period. Usually, but not always, a coincident tachycardia was noted, and occasionally there occurred a moderate tachypnea. Such febrile reactions were observed in 57 per cent of the patients, more commonly following the first dose of serum, sometimes in connection with all, occasionally with increasing severity as the course of treatment progressed. Arthritic reactions were noted in 53 per cent of the patients in whom arthritis was the predominant manifestation. These appeared within twenty-four hours of the injection, and consisted either in exacerbations in joints already involved, or in spreads to joints previously unaffected. Such reactions have been described by Small, who states that they

TABLE IV

*Summary of Therapeutic Results**

	No. of patients.	S.C.A. serum.		V110A serum.		Scarlatinal antitoxin.		Totals.	
		Success.**	Failure.	Success.	Failure.	Success.	Failure.	Success.	Failure.
Chorea	1	0	1	..	..	..	..	0	1
Arthritis	12	2	3	0	5	1	1	3	9
Pericarditis	3	2†	0	1†	0	..	..	3†	0
Carditis	4	0	2	0	1	0	1	0	4
Totals	20	4††	6	1‡	6	1	2	6§	14

* Does not include Cases 1, 5, 12, 21, 22 or 23.

** The term "success" must be accepted with the qualifications expressed in the text.

† These patients received neocinchophen in addition.

†† Two of these patients received neocinchophen in addition.

‡ This patient received neocinchophen in addition.

§ Three of these patients received neocinchophen in addition.

attack particularly the smaller joints; in our experience, however, the larger ones have been affected about as frequently as the others. Usually there has been subsidence within twenty-four hours, though in Cases 14 and 18 the condition became progressively worse. In Cases 5 and 11 severe toxemia with delirium followed the use of S.C.A. serum. Less common manifestations have included the appearance of a new murmur, numerous ectopic beats, an increase in pulmonary moisture (râles), and chill, vomiting and other phenomena of protein shock. Dosage of serum, whether large or small, has seemed immaterial; in fact, one of the most alarming reactions occurred three hours after an initial injection of 15 cc. of S.C.A bovine (Case 11), while in another case delirium of three days' duration set in following the use of 3 cc. of S.C.A. concentrated equine (Case 5). In the latter instance a full course of 10 cc. was given over a period of three days, and large doses of neocinchophen were administered; in addition to the delirium, severe febrile and pulse reactions occurred after each injection of serum, and numerous ectopic beats were observed. A dry pericardial rub of forty-eight hours' duration was

TABLE V

*Reactions: Tabulated According to Sera**

	S.C.A. serum.	V110A serum.	Scarlatinal antitoxin.	Totals.
Total doses	31	16	11	58
Temperature	17	5	2	24
Pulse	17	4	2	23
Respiration	5	0	2	7
Cyanosis	1	2	0	3
Delirium	2	0	0	2
Arthritis	5	6	4	15
Shock and chill	1	0	0	1
Ectopic beats	2	0	0	2
Restlessness	0	1	0	1
Dyspnea	0	1	1	2
Vomiting	0	0	1	1
Pulmonary signs	2	0	1	3
Gallop accentuated	1	0	0	1
New murmur	1	0	0	1

* Does not include Cases Nos. 12, 22 or 23.

noted in Case 10, though it did not appear until three days after the last dose, and consequently cannot fairly be attributed to the serum. Tables V and VI summarize these by-effects. Reactions occurring in "serum desensitized" patients have not been included, as the effect of the factor of serum allergy could not be controlled. As warning against serum treatment of such sensitive individuals may be cited, however, a severe asthmatic attack with deep cyanosis in one patient (Case 23), while in another instance severe carditis, with pericarditis and effusion, developed immediately (Case 22).

TABLE VI

*Reactions: Listed According to Principle Condition**

	Chorea.	Arthritis.	Pericarditis.	Carditis.	Totals.
Number of patients	1	15	3	4	23
Temperature	1	8	2	2	13
Pulse	1	8	2	1	12
Respiration	0	3	1	0	4
Cyanosis	0	1	1	1	3
Delirium and toxemia	0	1	1	0	2
Arthritis	0	8	1	0	9
Shock and chill	0	0	0	1	1
Ectopic beats	0	1	0	0	1
Restlessness	0	0	0	1	1
Dyspnea	0	1	0	0	1
Vomiting	0	0	0	1	1
Pulmonary signs	0	3	0	0	3
Gallop accentuated	1	1	0	0	1
New murmur	0	1	0	0	1
Patients without reaction—under drug	...	...	1	...	1
Patients without reaction—not under drug	...	2	...	1	3

* Does not include Cases Nos. 12, 22 or 23.

The occurrence of reactions seems to bear no relationship to subsequent improvement or to its permanency.

The danger of such reactions may perhaps be decreased by the simultaneous use of salicylates, which, however, as pointed out above, scientific precision usually forbade during this study. In the few instances cited, in which the condition of the patients seemed to prohibit delay, these drugs have rarely proven efficacious in warding off the reactions, and on one occasion truly alarming symptoms developed (Case 5).

Discussion. Generalizations based on a comparison of the three sera used in this study are hardly justifiable in so small a series of cases. The three different sera were made in as many different laboratories by different methods of immunization, and it is not improbable that had 3 horses or cows been immunized with the same strain of streptococcus by different methods similarly varying results would have been obtained. Hence it is probably better to disregard the effects from any particular serum and discuss them all together. It is, however, noteworthy that comparable symptoms, both favorable and unfavorable, were observed following all three types of serum; and it is not without interest that similar effects were observed over twenty-five years ago by Menzer,[5] Schmidt[6] and Sinnhuber,[7] when a polyvalent antistreptococcus serum prepared by the former was employed. All of these observations lead to the conclusion that any beneficial therapeutic effect observed must be dependent upon some other factor than a specific antitoxin against a hypothetical toxin elaborated by one strain of streptococcus.

Indeed, Menzer,[8] although for years an active proponent of a specific antitoxic action of serum, later attributed the beneficial effect to nonspecific protein therapy ("Reiztherapie"), and hence gave up the use of serum in favor of streptococcus vaccines. His abandonment of serum would appear one of the strongest arguments against its use. But the enthusiastic reports of Baruffaldi,[3] and the observations of Toogood[9] and Barach[10] with antiscarlatinal serum, together with Small's claims, made necessary a further study, with the results as above recorded.

From a theoretic viewpoint it may be asked: were any effects, either beneficial or otherwise, constantly attendant upon the application of these sera, which might be ascribed to their antistreptococcus qualities rather than to their actions as foreign proteins? In the absence of a control group treated simply with normal horse or cow serum a satisfactory answer to this question cannot be given.

Small[11] bases part of his hypothesis of the pathogenesis of rheumatic fever upon focal reactions in the small joints, together with general reactions following serum treatment. He attributed these reactions, which he designates as exudative, to a union of streptococcus antiprotein, contained in all antistreptococcus serum, with streptococcus protein, which he thinks is present in the tissues. Similar focal reactions have been reported following tuberculin treatment[12] and other forms of nonspecific protein therapy; hence this hypothesis demands further testing.

From another theoretic viewpoint one would not expect marked specific beneficial action to follow antistreptococcus serum therapy in rheumatic fever, for it is not of the general type of disease which responds favorably to serum. Usually rheumatic fever is not an acute disease like diphtheria, tetanus, or scarlet fever, but a subacute or chronic infection resembling tuberculosis or syphilis in many of its features.[13,14]

If a hypothetic toxin or some peculiar bacterial fraction or autolysate is responsible for the various manifestations of the disease, these substances are elaborated in varying amounts and intensity over long periods; hence it is improbable that a serum effect, which at best can be of only short duration, would be ultimately successful. Indeed, Small early recognized this possibility and recommended, in addition, a form of active immunization by repeated subcutaneous injections of an extract of Streptococcus Cardioarthritidis. He has reported better results with the use of this combined method than with the serum alone.

But in spite of these theoretic objections, if consistent improvement followed the application of antistreptococcus serum, one would be compelled to modify his theory, and utilize the serum. Our observations, together with those of Hill[15] and Wilson,[16] however, lead to the conclusion that antistreptococcus serum has no permanent antirheumatic influence, and that acute antipyretic, antiarthritic and antitoxic effects can be more certainly obtained from salicylates or neocinchophen. Whether the favorable effects which followed a combination of these drugs with serum in a few cases of pericarditis were anything more than fortuitous is open to discussion.

Of this one can be certain: that antistreptococcus serum in no way replaced the long-established therapy of rheumatic fever, nor does it apparently add enough to warrant its universal adoption. In our experience the unpleasant reactions sometimes attendant upon its application have not been outbalanced by a reciprocal certainty of therapeutic benefit.

Case Reports. CASE 2.—G. H., male aged seven and a half years, admitted March 5, 1928, to the service of Dr. Rivers because of chicken pox developing during the course of rheumatic carditis present at least since November, 1927. March 9, following the subsidence of the varicella, a further rheumatic cycle appeared. By March 14 there were present: temperature 104°, pulse 130, respirations 50, gallop rhythm, mitral and aortic regurgitation, positive Broadbent sign, severe precordial pain and hyperesthesia, hepatic enlargement with severe right upper quadrant pain. On this day S.C.A. concentrated equine serum was given intramuscularly in 3 doses of 10, 8 and 10 cc. respectively, between 12 noon and 9 P.M. No immediate reaction occurred. March 15 no evidence of improvement; similar serum was given, 15 cc. at 10 A.M., and 10 cc. at 6 P.M. During this day the temperature declined to 101.2° and the respiration to 40; no change in pulse rate. During the next three days no improvement; but dullness, bronchial breathing and bronchophony appeared at the angle of the left scapula, and there was cough with mucoid sputum. Neocinchophen therapy was instituted, and resulted in temporary improvement in the cardiac and pulmonary conditions.

CASE 4.—S. E., male, aged six years, admitted February 15, 1929, on the third day of his second attack of rheumatic fever; temperature, 102.4°; pulse, 130; respirations, 24; moderately severe polyarthritis, tonsillar infection, enlargement of heart to left, mitral systolic murmur heard throughout left axilla and left back, accentuated and split P_2, white blood cells 22,000. Under expectant therapy, temperature and pulse remained elevated; February 18, spreading of arthritis and a questionable gallop; 2.5 cc. of S.C.A. concentrated equine serum was given intramuscularly at 2 P.M. At this time temperature was 104.4°, pulse 130, and these levels were maintained throughout the day. February 19 A.M. the arthritis was much more severe; no new joints involved; there was a loud gallop; 2.5 cc. of the same serum was given at 11 A.M. Five hours later the temperature had risen from 103.4° to 104.4° and the pulse from 106 to 128. These levels were maintained throughout the day. The respirations rose to 60. February 20 A.M. the temperature was 102.4°, pulse 110, respirations 36, but during the day rose to 104°, 130 and 62, respectively. Arthritis considerably improved. February 21, fever much the same, though the pulse failed to pass 110° or the respirations 42; the gallop persisted; the patient was free from arthritis, but after a further two days this reappeared and the following morning (February 24) was so severe that neocinchophen, administration was begun. At no time during this interval did the temperature fall below 102°, and the pulse constantly ranged from 104 to 120 with the gallop unchanged. At the time of the arthritic relapse the pulse rate increased rapidly, the area of cardiac dullness increased, the murmur became harsh and grating and the cardiac action became quite tumultuous. The respiratory rate rose to 70, with grunting expiration, and fine râles were heard in the left chest posteriorly. There were no signs of consolidation either by direct examination or by Roentgen ray. The carditis and pneumonitis were at their height February 28, nine days after the completion of the serum course. Four days later a small crop of subcutaneous nodules appeared. The carditis ran a moderately severe course with subsequent marked improvement.

Case 7.—R. M., male, aged eighteen years, admitted January 2, 1929, on the thirteenth day of his first attack of rheumatic fever; temperature, 105°; pulse, 120; respirations, 36; cyanosis, severe toxemia, mild polyarthritis, small adherent tonsils, precordial hyperalgesia, cardiac enlargement, heart sounds muffled at the apex and over the precordium, loud pericardial friction rub, dullness in lower left axilla, also over the left lower lobe posteriorly merging into flatness at the base, with decreased tactile fremitus, bronchovesicular breathing and numerous moist and fine râles, merging into suppression at the left base, Grocco triangle to the right; roentgenograms indicative of pericardial and pleural effusions. Because of the critical condition neocinchophen was administered; this resulted in a moderate but very fleeting improvement; by January 11 the condition was very serious, and because of intolerance it had become necessary to reduce greatly the dosage of drug. At 2.45 P.M. of this day 5 cc. of S.C.A. concentrated equine serum was given intramuscularly. No reaction occurred. At 2 P.M. temperature was 102.8°, pulse 120; these levels were maintained until 8 P.M., after which a steady decline ensued. January 12 noon, temperature was 99.8°, pulse 104. No subjective or objective improvement was apparent. At 10.45 A.M. 5 cc. of serum was given. By 4 P.M. temperature had reached 103.4°; at 8 P.M. the pulse was 128. Shortly thereafter a decline in both occurred similar to that of the day before. January 13, distinct exacerbation of the polyarthritis; otherwise the general condition was the same, except that the steady loss of weight was checked. Thereafter steady improvement occurred, which by January 18 seemed complete, except for persisting leukocytosis and a small pleural effusion. The latter disappeared following a sharp febrile episode between January 28 and February 1. Drug withdrawal a few days later was followed by uneventful recovery.

Case 9.—C. K., male, aged thirty years, admitted May 9, 1929, on the fortieth day of the first attack of rheumatic fever; temperature 104°, toxemia, moderately severe polyarthritis, tonsillar infection, short systolic murmur along the left sternal border, palpable spleen. During five days control period fever continued, arthritis spread rapidly, the heart sounds became progressively softer and more distant, and there was steady loss of weight. On May 14, fever slightly lower but arthritis again spreading; 15 cc. of S.C.A. unconcentrated bovine serum was given intramuscularly at 2 P.M. No detectable reaction; the maximum temperature, 102.4° at 6 P.M., was only 0.6° higher than at 2 P.M., May 15, temperature had dropped to 100.2°, the arthritis was much improved and the heart sounds were clearer and louder. At noon 15 cc. of serum was given. At 8 P.M. a sharp rise in temperature to 101.8° occurred, thereafter an equally sharp decline. May 16, the arthritis had largely disappeared, heart sounds quiet and strong, murmur faint, temperature 100.2°. At 2.10 P.M. 20 cc. of serum was given. At 8 P.M. the temperature rose sharply to 101°, then quickly fell. May 17, arthritis in the right arm worse, and by evening the temperature was 101°. On May 19 the temperature once more began to rise and arthritis reappeared; by May 24 the patient was helpless, toxic, rapidly losing weight, with fever above 103°. Neocinchophen promptly controlled the fever and arthritis, and on its discontinuance on June 3 only a moderate shoulder stiffness persisted.

CASE 10.—F. P., male, aged thirty-three years, admitted December 5, 1928, on the fourteenth day of the first attack of rheumatic fever; temperature 103.4°, pulse 104, severe and widespread polyarthritis, ragged tonsils, reddened anterior pillars, palpable tonsillar nodes, soft systolic murmur at apex; leukocytes 27,000. During forty-eight hours of expectant treatment he became steadily worse, temperature hovering at 104° and pulse at 120; *P-R* interval 0.21. December 7, precordial tenderness and gallop rhythm. At 2 P.M. he was given intramuscularly 20 cc. of S.C.A. unconcentrated bovine serum without demonstrable reaction. A steady decline in fever occurred reaching 101.6° at 10 A.M. the following day. Pulse rate and arthritis unaffected, precordial tenderness still present, gallop rhythm less definite; *P-R* interval 0.20. December 8 at 12.15 P.M. he was given 20 cc. of the same serum without demonstrable effect. December 9 fever ranged between 101.4° and 102.4°, pulse 105 to 115, arthritis slightly improved, gallop doubtful, patient less toxic. December 10, however, definite improvement appeared, and by December 16 the temperature was normal, only arthritic symptoms were tender spots about the larger joints; *P-R* interval 0.18, white blood cells 10,640. Precordial tenderness and gallop had disappeared. The periarticular tenderness persisted until after tonsillectomy; otherwise the patient's course was satisfactory. On December 11 and 12 there was a recurrence of precordial tenderness and a dry-friction rub was heard at the left sternal border at the level of the second and third interspaces.

CASE 11.—M. F., male, aged twenty-two years, admitted June 3, 1929, during the twelfth week of the first attack of rheumatic fever; temperature 100.2°, pulse 120, mild polyarthritis, chronic tonsillar infection, systolic precordial heave, systolic impulse and thrill in second left interspace, cardiac enlargement, precordial pain and tenderness, widely transmitted apical systolic murmur, short mid-diastolic puff medial to apex, systolic murmur at base heard in neck vessels. P_2 accentuated and split, systolic and diastolic crunching sounds at level of second interspace, scattered subcutaneous nodules. White blood cells 9,800. During following week he became steadily worse. June 10, pulse 152, widespread erythema multiforme, mushy and muffled first sound, pericardial friction rub, increase in the size of the heart, bronchovesicular breathing and bronchophony at the angle of the left scapula, white blood cell 18,800; increased weight associated with fluid retention. Because of severity of symptoms neocinchophen was started in full doses without inducing antipyresis or amelioration of symptoms in forty-eight hours. June 12, 15 cc. of S.C.A. bovine unconcentrated serum intramuscularly at 11.30 A.M. At 8 A.M. of this day the temperature had been 101.4°, pulse 136; at noon, temperature 102°, pulse 148; at 2 P.M., temperature 103°, pulse 152. By this time the patient was delirious, restless and required restraining; head retracted and head and eyes turned constantly to the right, violent muscular tremors, heart sounds soft and distant, pulse of poor quality, cyanosis of the extremities. No signs of congestive failure. During the day 0.7 gm. digitan was given, and the dosage of neocinchophen was reduced. By evening temperature and pulse were falling sharply and delirium had vanished. June 13 condition improved, diminution in cardiac size, no precordial pain and little tenderness, no signs of compression of the left lower lobe, sticky râles at both bases; 7.5 cc. of serum was given at 10.30 A.M., and at 3.30 P.M. At 10 A.M. temperature was 100.5°, pulse 132; maximum temperature at 2 P.M., 101.8; pulse 136; no untoward reaction. During the night there was a sharp drop in temperature and pulse, which on the following day failed to pass 100.8° and 110 respectively. June 14, 10 cc. of serum was given at 11.20 A.M. and at 3.30 P.M. without demonstrable reaction. Steady and rapid improvement continued. Drug withdrawal on June 16 was uneventful, and the further course was entirely satisfactory.

CASE 17.—A. P., female, aged eight years, admitted January 10, 1929, on the seventh day of her second attack of rheumatic fever; temperature, 105°; pulse, 140; respirations, 44; marked orthopnea, acute polyarthritis, scarred remnants of tonsillar tissue, anterior pillars injected, tonsillar nodes enlarged and tender, diffuse precordial pulsations, cardiac enlargement, loud pericardial friction rub with synchronous thrill, gallop rhythm; over the left lower lobe dullness with bronchial breathing and bronchophony merging into suppression at the base, diminished breath sounds at the right base, white blood cells 33,600; roentgenogram suggests pericardial effusion. Under neocinchophen therapy steady improvement occurred for a week, with disappearance of arthritis, lowering of temperature, pulse rate and leukocyte count, and retrogression of pulmonary and precordial signs. Complete recovery was not achieved, however, and on January 21 an obvious relapse was under way in spite of drug administration. On this day 10 cc. of V110A unconcentrated bovine serum was given intramuscularly at 3 P.M. without immediate reaction. The temperature peak for the day was 102.8° at 4 P.M. followed by a steady decline; pulse little affected. January 22, patient appeared rather better; 10 cc. of the same serum was given at 2.40 P.M. No demonstrable reaction occurred. The temperature peak for this day was 100.5°; pulse ranged between 120 and 140. Except for a rise in temperature to 102.8° on the evening of the twenty-third the course during the ensuing two weeks was one of steady improvement, with disappearance of all signs of pericarditis and pulmonary involvement, decline in tachycardia, decreasing leukocytosis, diminution in cardiac area, and normal or only very slightly elevated temperature. Drug withdrawal at the end of this period was uneventful. The patient entered then upon a prolonged subfebrile course, free from acute episodes.

CASE 19—W. F., male, aged forty-one years, admitted February 20, 1929, on the twenty-fourth day of the first attack of rheumatic fever; temperature 100.6°, pulse 90, severe polyarthritis, congested fauces and pharynx, short systolic murmur at the base to left and right of the sternum, soft, diastolic murmur in 2d, 3d and 4th left interspaces; blood pressure 115 systolic, 40 diastolic; white blood cells, 17,400. During the next forty-eight hours the temperature rose to 103.8° and there was spreading arthritis. February 22 at 11.30 A.M. he was given intramuscularly 20 cc. of V110A unconcentrated bovine serum without demonstrable effect on fever or pulse. February 23 at 8 A.M. temperature was 101.8°, pulse 88; the arthritis had ceased to spread but was more severe in the right hand; 20 cc. of serum at 11.30 A.M. During the day the temperature rose to 103.8°, the pulse to 100. February 24, the temperature was 100.8°, pulse 90. Most of the previously involved joints were improved; on the other hand, a few were worse and there was reinvolvement of joints which had been free for some time. During the day the temperature rose rapidly to 103.6°, to fall sharply during the night. February 25, there was considerable improvement, except for the newly involved joints of the day before, in which the process was at its height; February 26, these in turn had begun to yield and general improvement was quite marked. The temperature never became normal, however, and during the period February 27 to March 2 the febrile course was renewed; widespread and severe polyarthritis developed, and rapid loss of weight occurred. Neocinchophen therapy was instituted upon the latter day and a very prompt and gratifying improvement ensued. Except for a mild relapse attendant upon premature withdrawal of drug, the remainder of the patient's course was uneventful.

CASE 25.—N. S., male, aged forty-three years, admitted April 8, 1929, on the sixteenth day of his fifth attack of rheumatic fever; temperature 102.8°, pulse 92, moderately severe polyarthritis, chronic tonsillar infection, systolic murmur at apex poorly transmitted outward, systolic murmur in second left interspace, radial arteries slightly thickened, blood pressure 152 systolic, 85 diastolic. During a control period of ten days the disease was irregularly continuous, temperature 100.6° to 103.6°, pulse 90 to 100, arthritis never absent and at times very severe, steady loss of weight and strength. April 19, 3 cc. of concentrated scarlatinal antitoxin was given intramuscularly at 4.15 P.M. without immediate reaction. At 4 P.M. temperature was 101.6°; at 6 P.M., 102°; it declined steadily during the night. Similar declines had occurred nightly during the control period, so that the A.M. temperature of 100° was no lower than usual. The pulse hovered around 110°, about 6 points higher than usual. The morning of April 20 there was a severe exacerbation of the arthritis, with spreading to the hips and knees. At 10.45 A.M. 3 cc. of antitoxin was given. There was no reaction, and during the day the temperature failed to surpass 101°. April 21, arthritis considerably improved, and fever did not exceed 100.6°. The weight loss was checked. April 22, the relief was more pronounced; 4 cc. of the antitoxin was given at 2.30 P.M. without reaction; temperature not above 100°; pulse range 110 to 120. Improvement continued for three days; there was then a five-day cycle of serum sickness with small-joint arthritis and urticaria. Following the spontaneous subsidence of this episode the further course was entirely satisfactory.

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PSITTACOSIS

REPORT OF A CASE

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Within the period of a year approximately 500 cases of psittacosis have been recorded.[1] The clinical picture of the disease has been recognized for at least fifty years,[2] and many of the recent cases have been carefully described. Furthermore, the literature has been excellently reviewed by a number of workers.[3] The reasons for reporting the present case are the facts that (1) daily blood counts were made by means of the supravital method of staining; (2) the changes in the lungs were followed by daily roentgen examinations; (3) electrocardiographic studies were made. and (4) a positive laboratory diagnosis was obtained through the use of white mice.

Work on psittacosis was begun in our laboratory February 26, and has continued for four months. A large number of parrots, mice, guinea-pigs, rabbits and monkeys have been inoculated with the virus, and the course of the infection in these animals has been under investigation. All of this work - even the housing of the infected animals - has been conducted in the center of a large research institution. In fact, our laboratory is the most centrally located one in the institute, as it is near the business manager's offices, the library, the dining rooms, the central medium room and the girls' rest rooms. In spite of the infectious nature of psittacosis, we have been able to prevent the occurrence of any cases among the workers and employees. Regardless of the care taken to prevent a spread of the infection, however, one of the physicians (G. P. B.) who came in very close contact with

[1] Roubakine, A.: Monthly Epidemiological Report of League of Nations 9: 141, 1930.

[2] Ritter, J.: Deutsches Arch. f. klin. Med. 25: 53, 1880.

[3] Roubakine (footnote 1). Hutchison, Robert; Rowlands, R. A., and Simpson, S. L.: Brit. M. J. 1: 633 (April 5) 1930.

the animals and infectious materials contracted psittacosis five weeks after the inception of our studies. The mode and time of infection are not known.

Report of Case

History.—G. P. B., aged 31, gave a family history and past history that are unimportant. The onset of the present illness was acute, beginning about 5:30 p.m., March 31, with headache, backache and general muscular pains. The symptoms increased during the evening. Sleep was broken by a slight chill at 5 a.m., April 1. The headache was general at first but soon became basilar, and severe pains radiated down the spine to the lumbar region. Photophobia, burning behind the eyeballs, anorexia and nausea appeared, April 1. In spite of feeling very sick, the patient said nothing of his condition and worked until 4 p.m., when he had two shaking chills, after which his temperature rose to 102 F. At this time he reported his condition and was admitted to the hospital. On admission his temperature was 101.2 F., pulse 90, respiration 22, white blood cells 13,200. Physical examination revealed nothing more than suffusion of the eyeballs, injection of the mucous membranes of the throat, and a heavily coated tongue.

Course in the Hospital.—The temperature, pulse and respirations are shown in the accompanying chart. The temperature was taken by mouth.

April 2, the patient had a restless night because of the headache and backache, which were not relieved by the administration of ordinary analgesics and morphine. The eyes were still red, the throat was injected and the tongue was white. An examination of the chest and the abdomen was negative. A roentgenogram of the chest, however, revealed a small shadow on the left side just below the level of the base of the heart (fig. 1 *A*).

April 3, the headache and backache were still pronounced; the tongue was coated and the breath foul. The lungs showed diminution of tactile fremitus and breath sounds on the left side laterally and posteriorly. A roentgenogram revealed a definite shadow on the left side involving the middle and lower third of the lung. The shadow was not dense but was more marked and extensive than that of April 2. The abdomen was tender and distended.

April 4, on percussion there was dulness in the left axilla and over the lower posterior part of chest. Over these areas, diminution of tactile fremitus and breath sounds was found. The character of the breath sounds was not altered, but posteriorly showers of fine râles were heard on deep breathing.

April 5, the headache was less severe. Distention of the abdomen was still marked. There was no pain in the lungs, but the patient had a slight, unproductive cough. Respirations were not rapid. Tactile fremitus was almost absent over the left side of the chest anteriorly. Beneath the angle of the left scapula there was an area the size of one's hand over which dulness was marked, and distant tubular breathing accompanied by fine râles was heard. Tactile fremitus and voice sounds, however, were not increased. The roentgenogram showed the shadow on the lung denser and more extensive than on the day before (fig. 1 *B*).

April 7, headache was still present, and the tongue was white. There was no stomatitis. The slight, unproductive cough persisted. There was no change in the signs in the lungs. The abdomen was still distended and tender, and the liver somewhat enlarged and tender. The spleen was not palpable.

April 8, one mass of grayish yellow tenacious sputum was brought up. No change in the condition of the lungs was noted.

April 10, a drenching sweat occurred during the night, as it had for the past three nights. The patient raised one mass of yellow, blood-tinged tenacious sputum. The signs in the chest were less marked. A roentgenogram showed the shadow on the lung to be clearing (fig. 1 *C*).

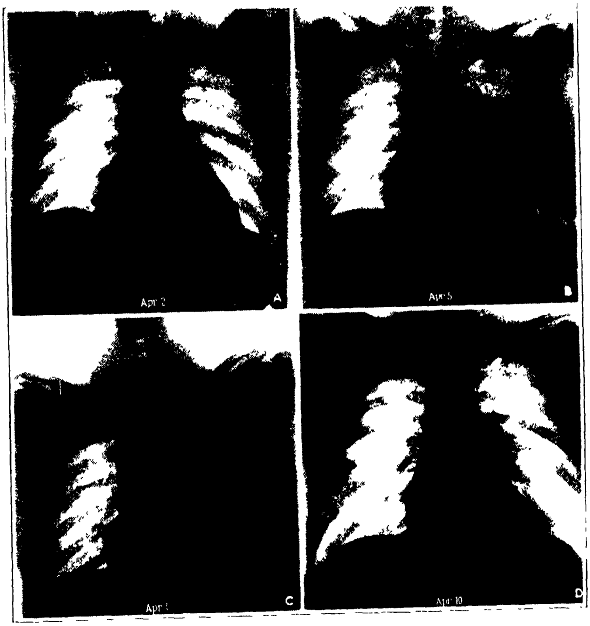

FIG. 1. *A*, small shadow at left hilus near base of heart; *B*, extensive involvement of left lung; *C*, beginning clearing of lung, from periphery inward toward hilus; *D*, resolution has been extremely rapid; note only the residual hilar shadow.

April 12, the general condition of the patient was improved, but abdominal distention was still annoying. On examination, the signs in the lungs were found to be disappearing rapidly. A roentgenogram showed a shadow similar to that seen on April 2. This shadow persisted for several weeks and then gradually disappeared (fig. 1 *D*).

Convalescence.—April 15, the temperature for the first time did not go above normal. Convalescence was very slow. A marked wasting of the muscles, particularly those of the legs, induced an abnormal gait that endured for nearly a month.

Laboratory Observations.—Roentgenographic Examinations: Dr. Ross Golden of the Presbyterian Hospital, who kindly examined the roentgenograms of the lungs, reported that the films showed an interesting development and regression of a pneumonic process about the left hilus. The development was steady from April 2 until April 5. The film on April 6 showed an apparent diminution in the shadow. This might have been due in part to the fact that this film was somewhat denser than that taken on the previous day, as there was no evidence of diminution in the shadow on the 7th. The film taken on the 10th, however, showed a striking regression of the pneumonic process. This regression continued until April 25, when the last film was taken. At this time, there remained only an enlarged hilus shadow with infiltration around some of the bronchi radiating out to the left lateral chest wall. These films do not present the picture of an ordinary bronchopneumonia, which is more likely to be located along the descending bronchi or toward the apex. It is the picture usually encountered in influenzal pneumonia. There was a hazy indefinite shadow through which could be seen the somewhat exaggerated lung markings. The process obviously developed from the hilus outward and regressed from the periphery inward. Dr. Golden stated that if these films had been presented to him without a history, he would have interpreted them as a bronchopneumonia of the influenzal type. He did not know of any reason, however, why such a process should not be encountered also in psittacosis.

Blood: Blood counts were made throughout the course of the illness and for more than two weeks during convalescence. Differential counts were made on preparations supravitally stained with neutral red and janus green according to the method of Sabin.[4] The results are summarized in the accompanying table. The first count, made twenty-four hours after the onset of the illness, showed a moderate leukocytosis. Following this, the cells decreased in number and by the end of the first week had reached their lowest level (6,000). On the ninth day of the disease the count rose sharply and thereafter maintained a level of about 8,000 to 9,000. The polymorphonuclear neutrophils showed a striking "shift to the left," i.e., a number of young forms, beginning on the fourth day of the disease. The maximum change was reached on the ninth day, when the absolute number of polymorphonuclear cells was at its lowest level. At this time there were 31 per cent of band forms and 1 per cent of myelocytes. Eosinophils were entirely absent during the first few days of the illness, as is the case in many acute infections. Lymphocytes remained low throughout. On the fourth day they constituted only 5 per cent of a total count of 9,000. The number of these cells rose sharply for the first time two days after the temperature became normal. Such a moderate lymphocytosis, coincident with recovery, has been noted in other acute infections.

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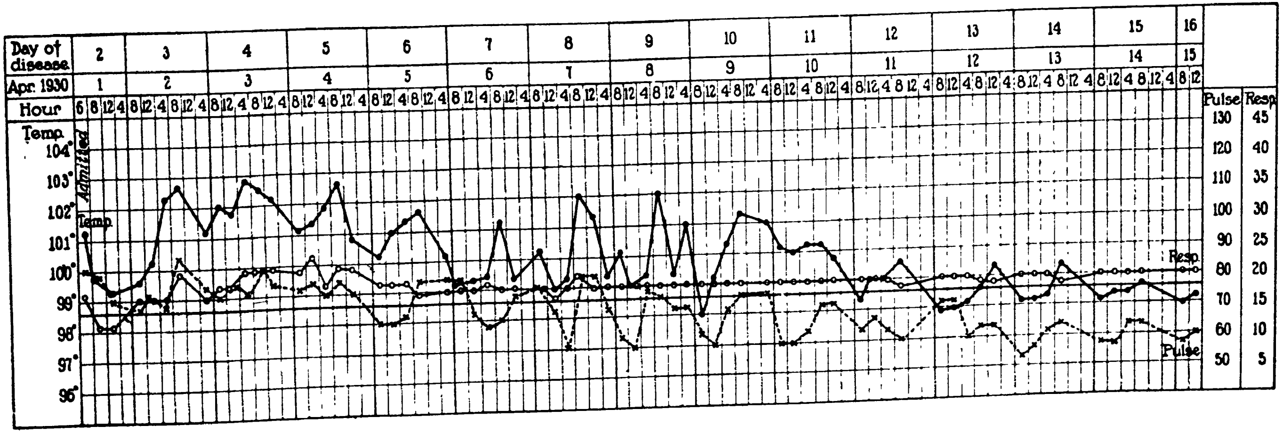

FIG. 2.—Temperature, pulse and respiration in a case of psittacosis. The disproportion between the pulse and the temperature is obvious. In spite of the involvement of the lungs, the respirations were slow.

The monocytes showed nothing of great importance. The supravital technic, which makes it possible to identify these cells with certainty, accounts for the high numbers of monocytes that were constantly present. It may be noted that at no time were the monocytes "stimulated" or changed in appearance, as is the case in tuberculosis. Toward the end of the febrile period, however, a slight monocytosis occurred. The blood picture showed only changes that one might expect in a number of acute infectious diseases that tend to produce a mild leukopenia.

Urine: A moderate amount of albumin was present in the urine at the height of the illness. There was distinctly more than one might expect from fever alone. In other respects the urine showed nothing of importance.

Blood Pressure: The lowest level was reached, April 13, when it was 98 systolic and 68 diastolic. By April 22 it had returned to 118 systolic and 68 diastolic.

Electrocardiograms: During the course of the disease, electrocardiographic examinations were made, but in spite of the relatively slow pulse nothing particularly abnormal was noted.

Blood Culture: Blood drawn April 2 was tested for its sterility in flasks of meat infusion broth and also by the plate method. All of the preparations remained sterile for ten days.

Stool Culture: A culture was made on Endo's medium from a stool passed on April 10. No non-lactose fermenting bacilli were found.

RESULTS OF ANIMAL INOCULATIONS

Five cubic centimeters of blood, drawn April 2, was injected intramuscularly into an Amazon parrot. Blood was also placed in the bird's drinking water and on its food. The parrot remained well and when reinoculated with psittacosis virus six weeks later was found to be susceptible.

Blood drawn April 2 was injected in 0.5 cc. amounts intraperitoneally in each of four mice. They remained well for six weeks, after which time they were discarded.

Nasal washings were collected from the patient, April 2. A portion of this material was dropped into the nose and mouth of an Amazon parrot. The remainder, 10 cc., of the washings was placed in the bird's drinking water. The parrot was not sick at any time, and six weeks later on reinoculation with psittacosis virus was found to be susceptible.

April 10, the patient raised a mass of yellow blood-tinged tenacious sputum. This was washed several times in sterile Locke's solution and then emulsified in Locke's solution. A culture was made on blood agar of a drop of the emulsion, and the predominating organism was found to be *Streptococcus viridans*. One cubic centimeter of the sputum emulsion was injected intraperitoneally in each of five mice. One of the mice died during the second night following the inoculation. Passage from this animal was not attempted, as postmortem changes were marked. Another mouse died fifteen days after inoculation, and from the liver and spleen of this animal an emulsion was made which was injected (0.5 cc.) intraperitoneally in each of four mice. These animals became sick and died within seven days. By means of liver and spleen emulsions from sick or dead mice the virus has now been carried through nine passages. It regularly kills mice within from four to seven days. Furthermore, an Amazon parrot inoculated intramuscularly with an emulsion of liver and spleen from one of these mice developed a typical attack of psittacosis. Cultures of the livers and spleens have revealed no ordinary anaerobic or aerobic bacteria. Moreover, in smears made from the liver and spleen are found the very minute bodies described by Levinthal,[5] Lillie[6] and Coles.[7] Sections of the liver show fatty degeneration and areas of focal necrosis. The picture of the disease caused by this strain of the virus is identical with that produced by two other strains of psittacosis virus being investigated in the laboratory.

COMMENT

The picture presented by this case of psittacosis was typical. The onset of the illness and the general appearance of the patient reminded one of a mild attack of yellow fever in which jaundice and bleeding are absent.[8] (Some have likened it to typhoid.) Nevertheless, the important observation on physical examination is a consolidation of the lungs.

The chief symptoms were severe and persistent headache, backache, and abdominal discomfort caused by distention. The tongue was covered by a peculiar heavy white coat that endured for more than two weeks. From the chart it is obvious that there was a disproportion between the pulse and the temperature. The most striking feature of the disease was the fact that, in spite of the marked involvement of the left lung, no symptoms referable to the chest occurred. There was no increase in rate of respiration, no pain on breathing, very little cough, and no expectoration except on two occasions.

Bedson and his co-workers,[9] by means of injections of patients' blood into love birds, have been able to demonstrate the presence of psittacosis virus. The injection of 5 cc. of blood from our patient into a susceptible parrot, however, yielded a negative result. Of course, this does not cast doubt on Bedson's results.

[5] Levinthal, W.: Klin. Wchnschr. 9: 654, 1930.

[6] Lillie, R. D.: Pub. Health Rep. 45: 773 (April 11) 1930.

[7] Coles, A. C.: Lancet 1: 1011 (May 10) 1930.

[8] We have recently cared for three patients with yellow fever and have been struck by the similarity between the two diseases.

[9] Bedson, S. P.; Western, G. T., and Simpson, S. L.: Lancet 1: 345 (Feb. 15) 1930.

Summary of Blood Counts

Date	Total White Blood Cells	Polymorphonuclear Neutrophils			Eosinophils		Basophils		Lymphocytes			Monocytes	
		Absolute No.	Total Per Cent	Per Cent Band Forms	Absolute No.	Per Cent	Absolute No.	Per Cent	Absolute No.	Per Cent Small	Per Cent Large	Absolute No.	Per Cent
4/ 1/30	13,200	10,296	78	5	...	..	132	1	1,320	8	2	1,452	11
4/ 2/30	7,400	5,476	74	4	...	..	74	1	1,332	16	2	518	7
4/ 3/30	9,000	7,650	85	11	...	..	...	..	450	3	2	900	10
4/ 4/30	10,100	8,383	83	24	...	..	...	..	808	6	2	909	9
4/ 5/30	7,400	5,032	68	27	74	1	...	..	1,702	18	5	592	8
4/ 6/30	7,700	4,697	61	16*	385	5	77	1	1,386	14	4	1,078	14
4/ 7/30	7,300	4,599	63	20	73	1	...	..	1,679	21	2	949	13
4/ 8/30	6,000	4,380	73	31†	60	1	...	..	960	14	2	540	9
4/ 9/30	11,600	9,280	80	10	348	3	116	1	580	4	1	1,276	11
4/10/30	9,700	6,984	72	12	388	4	97	1	679	6	1	1,552	16
4/11/30	10,300	6,489	63	14*	309	3	...	..	1,648	12	4	1,751	17
4/12/30	9,700	6,790	70	4	679	7	97	1	1,552	11	5	582	6
4/14/30	9,100	6,370	70	..	182	2	91	1	1,274	11	3	1,183	13
4/15/30	9,900	7,722	78	..	99	1	...	..	1,386	8	6	693	7
4/16/30	9,300	5,394	58	..	...	..	...	..	3,441	37	..	465	5
4/17/30	10,200	6,834	61	3	102	1	102	1	2,550	18	7	612	6
4/18/30	8,000	5,520	69	2	320	4	...	..	1,280	12	4	880	11
4/19/30	9,400	6,956	74	..	188	2	94	1	1,504	13	3	658	7
4/21/30	10,200	6,834	67	1	204	2	102	1	2,142	16	5	918	9
4/22/30	7,900‡	4,740	60	2	79	1	79	1	2,291	15	14	553	7
4/23/30	10,000‡	6,800	68	..	600	6	...	..	2,000	20	..	400	4
4/24/30	9,400				No differential on this date								
4/25/30	9,300	5,952	64	..	465	5	...	..	1,581	14	3	1,302	14
4/26/30	8,100	4,698	58	..	486	6	...	..	2,025	24	1	891	11
4/28/30	9,000	5,400	60	..	270	3	270	3	2,340	23	3	720	8
4/29/30	8,400	5,796	69	..*	420	5	168	2	1,176	10	4	756	9
4/30/30	9,700	6,014	62	..	582	6	...	..	2,037	20	1	1,067	11
5/ 1/30	9,200	4,508	49	1*	644	7	92	1	2,668	28	1	1,196	13
5/ 2/30	7,400	3,478	47	..*	370	5	74	1	2,368	32	..	1,036	14

* Plus 1 per cent metamyelocytes.

† Plus 1 per cent C myelocytes.

‡ Plus 2 per cent degenerating cells.

Krumwiede and his co-workers[10] have shown that mice are susceptible to the virus of psittacosis. For the first time, however, we have been able to demonstrate that these animals can be used for the diagnosis of the disease in human beings. This was accomplished by injecting washed sputum into their peritoneal cavities. Naturally, the presence of virulent pneumococci and hemolytic streptococci will interfere with this method. In case these organisms are present, the sputum should be emulsified and passed through a Berkefeld V filter before being used.

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[10] Krumwiede, C.; McGrath, M., and Oldenbusch, C.: Science 71: 262, 1930.

The results of our work in connection with the case reported at this time serve to emphasize the fact that the etiologic agent of psittacosis is neither Nocard's bacillus nor any other ordinary bacterium. Moreover, it appears that the white mouse may serve as a suitable animal for diagnostic purposes. Thus, in many instances, a laboratory diagnosis that ordinarily would be unfeasible will become possible. Finally, it has been shown that the virus of psittacosis is in the sputum of individuals with involvement of the lungs, and this fact should be borne in mind by those who care for these patients.

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PSITTACOSIS

OBSERVATIONS CONCERNING THE EXPERIMENTAL DISEASE IN PARROTS, MICE, RABBITS, GUINEA-PIGS AND MONKEYS

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In a previous paper[1] the fact that mice, rabbits and guinea-pigs are susceptible to psittacosis was reported. No description, however, of the pathologic changes observed or of the work on immunity was given, nor was any mention made concerning experimental psittacosis in monkeys. Furthermore, the portal of entry and exit of the virus was not discussed. The occurrence of a laboratory infection[2] in one of the workers (G. P. B.) made feasible certain experiments on immunity and also furnished a series of roentgenograms of the lung involvement to be used for comparison with those obtained of the experimental disease in monkeys. Therefore, in view of the general interest in psittacosis, it seems advisable at this time to record briefly the results of some of our work.

Neutralization of Psittacosis Virus with Convalescent Serum

Although serum from recovered individuals has been used in the treatment of psittacosis, no one has shown experimentally that such serum has curative or prophylactic properties. In fact, the outcome of one reported experiment[3] was doubtful. The results of our work also indicate that it is not a simple matter to demonstrate neutralizing properties in convalescent serum.

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[1] Rivers, T. M., and Berry, G. P.: Proc. Soc. Exp. Biol. & Med. **27:** 802, 1930.

[2] Rivers, T. M.; Benjamin, Bernard, and Berry, G. P.: Psittacosis, this issue, p. 577.

[3] Armstrong, Charles; McCoy, G. W., and Branham, S. E.: Pub. Health Rep. **45:** 725 (April 4) 1930.

In the first experiments performed to show immune properties in convalescent serum, mice were used. Since the results were irregular, rabbits were chosen for further work. As previously reported,[1] by means of intracerebral inoculations of virus, psittacosis can be transmitted indefinitely from rabbit to rabbit. Only a few of the infected animals die, but all of them have a very high temperature, from

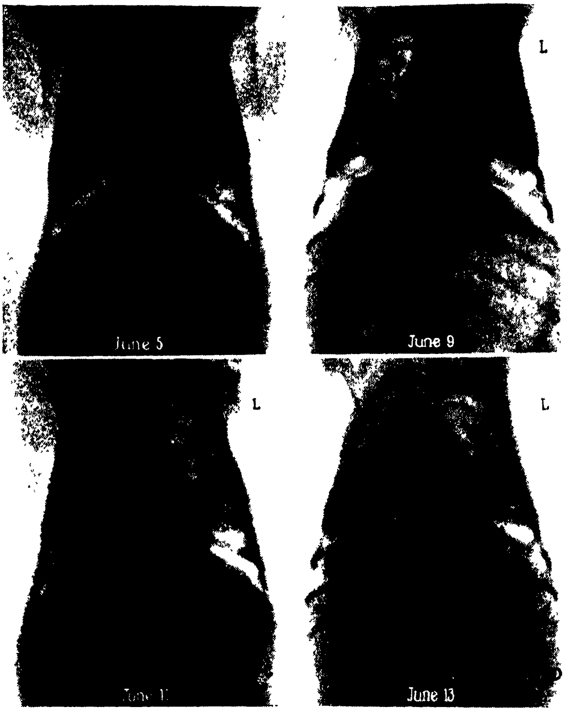

FIG. 1.—Monkey J.: *A*, two days before inoculation; *B*, two days after intratracheal inoculation with psittacosis virus, with initial lesions near the hilus; *C*, four days after inoculation, with lesions more extensive; *D*, six days after inoculation: Involvement of lungs extends to the periphery; heart shadow has definitely increased in size.

104 to 108.5 F. Therefore, the temperature was chosen as an index of neutralization. Specimens of serum collected from a normal adult and from the patient (G. P. B.) on the second day of the disease were used as controls. Serum taken from the patient thirteen days after his temperature had returned to normal was the test material. Properly graded dilutions of the virus were mixed with equal amounts of the different serums and incubated for five or six hours at 37 C.,

after which 0.25 cc. of each mixture was injected intracerebrally into rabbits. Daily observations of the temperatures were made for a week. The normal serum and the serum from the patient early in the disease did not prevent high temperatures (105–107 F.) in the animals, while some of the animals that received a mixture of virus and convalescent serum failed to show a temperature above 104 F.

Reinoculation Experiments

It has been presumed that animals which have recovered from psittacosis are immune to reinfection. Little experimental proof, however, has been adduced to substantiate such a presumption. We have been able to demonstrate that an infection with the virus does lead to immunity in recovered parrots and rabbits.

A number of mice recovered from an infection with psittacosis virus. When they were reinoculated, many of them died. Although they survived longer than did the controls, the results of the experiment were not very striking. Three parrots recovered from an experimental infection. When these animals were reinoculated intranasally with a potent virus that promptly killed the control bird, they showed no evidence of infection. Furthermore, a large number of rabbits intracerebrally inoculated with virus or with normal organ emulsions were allowed to recover. Then, from three to eight weeks after recovery, they were reinoculated with virus. All of the rabbits, with the exception of one, which showed evidences of infection (high fever) after the first inoculation, exhibited none on reinoculation. The animals that received normal organ emulsions as a primary inoculation, however, had high fever when reinoculated later with virus.

Portal of Entry and Exit of the Virus

In handling a highly infectious disease, it is desirable to know the portal of entry and exit of the virus in various hosts. Since the parrot and man are the hosts of particular interest, most of the discussion, either directly or indirectly, will be concerning them. Krumwiede[4] and his co-workers performed one experiment in which they were unable to demonstrate psittacosis virus in the fecal droppings of a sick parrot. Armstrong and his collaborators,[3] however, were able to infect birds with filtered fecal material. The results of our own work reveal that the virus is in the feces and also in a mixture of the materials collected from the nose, mouth and procrop of a sick parrot. Furthermore, parrots are readily infected by dropping the virus in the nose. From

[4] Krumwiede, Charles: Personal communication to the authors.

our work on monkeys, to be described later, one may conclude that the virus enters man by way of the nose or mouth. Moreover, from our experience with a human case of psittacosis,[1] it is obvious that the virus is in the sputum.

From a number of infected parrots we have collected uncontaminated feces. Intranasal instillations of emulsions of this material regularly produced psittacosis in susceptible birds. Infected birds that had been killed with chloroform were suspended by their feet. Frequently a thick starchy material ran from the mouth and nose. Much of this undoubtedly came from the procrop. When this material was instilled into the nose of susceptible birds, psittacosis was induced.

Pathology of Psittacosis in Parrots, Mice, Rabbits and Guinea-Pigs

Since the virus of psittacosis undoubtedly attacks the lungs of man, many workers have devoted most of their attention to changes found in the lungs of experimental hosts. In dealing with parrots, mice, rabbits and guinea-pigs, however, we have been struck by the fact that the lungs in these animals are only rarely involved. In parrots inoculated intramuscularly or intranasally, and in mice infected intraperitoneally, the most constant and characteristic changes are found in the liver and the spleen.

Parrot Liver.—Gross Pathologic Changes: The liver is usually larger than normal with rounded edges, and has a yellowish tint indicating fatty degeneration. Frequently its surface is studded with white dots of varying sizes some of which are surrounded by red areolae. At times large areas of the liver are yellow and are encircled by hemorrhagic zones. Such lesions affect the edges of the organ and suggest infarcts.

Microscopic Changes: The characteristic lesion of psittacosis in parrots consists of multiple, discrete areas of liver cell necrosis, distributed irregularly throughout the lobules but with a tendency to be more numerous near the edge of the organ. The condition appears to have its onset in the death of isolated liver cells or groups of cells; the cytoplasm becomes acidophilic and granular, and shrinks from contact with other cells. The nuclei become hyperchromatic and pyknotic, and eventually disappear completely. At this stage, mononuclear phagocytes and a few polymorphonuclear cells surround and infiltrate the lesion. As the process progresses, the necrotic liver cells disappear, leaving strands of acidophilic, hyaline material which may or may not show collections of leukocytes and depositions of fibrin. Often the areas of necrosis are so large that one is led to question whether they are the result of vascular thromboses. Striking alterations in the vascular channels are not usually seen, but in certain cases fibrin thrombi, involving the portal vessels, are found. In most cases only small vessels are occluded, but occasionally a large branch is involved. The question as to whether the areas of necrosis result from vascular thromboses or whether the thromboses are caused by the necrosis cannot be answered at present. When necrotic areas penetrate to the surface of the liver, accumulations of inflammatory cells are seen under Glisson's capsule and are doubtless the cause of the perihepatitis and peritonitis that are so often encountered.

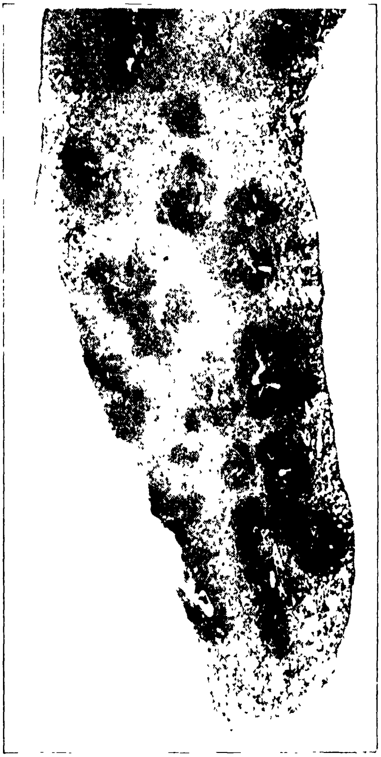

FIG. 2.—Section through a lobe of a monkey's lungs intratracheally infected with psittacosis virus: The peripheral portion of the lobe in places is still air-containing; the reaction is most intense around the bronchi and blood vessels; eosin and methylene blue; slightly reduced from a photomicrograph with a magnification of 6 diameters.

Throughout the organ there is a proliferation of Kupffer cells and focal accumulations of wandering mononuclear cells of the same general type. Some of these have a highly vacuolated cytoplasm containing masses of fat. Many plasma cells are also present.

The bile ducts show a variable degree of involvement. In cases in which only small lesions are present, no reaction in these structures is observed. In the presence of extensive destruction of the parenchyma, however, there is an extremely active proliferation of the bile ducts.

Parrot Spleen. Gross Changes: The spleen is always greatly enlarged and very friable.

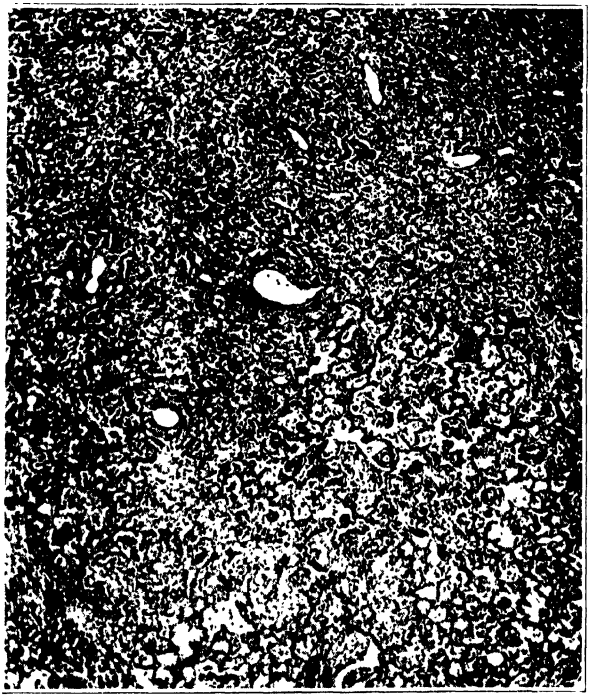

FIG. 3.—Consolidation in a monkey's lungs caused by psittacosis virus: The upper portion of the picture shows a disappearance of air-containing alveoli induced by a thickening of the alveolar walls and by a mononuclear exudate within the alveoli; in the lower portion, the thickened alveolar walls are still definable; eosin and methylene blue; slightly reduced from a photomicrograph with a magnification of 65 diameters.

Microscopic Changes: The normal architecture of the lymphoid follicles is almost completely obliterated, while the reticular and sinus structures are well preserved. The entire organ is densely infiltrated with wandering phagocytic cells which have a vacuolated cytoplasm containing amorphous débris, pigment and globules of fat. The increase in size apparently is due to the content of blood and the enormous number of mononuclear cells.

Mouse Liver. -Gross Changes: The liver is always larger than normal. Fatty degeneration is a prominent feature and at times is so marked that the whole organ becomes chamois colored. In addition to these changes, white or yellow dots often stud the surface. These lesions tend to be more numerous near the edge of the organ and may be surrounded by red areolae.

Microscopic Changes: Pathologic alterations in the livers of mice always occur and are extremely characteristic. A general infiltration with fine globules of fat and a granular degeneration of the liver cells are present. Isolated groups of cells, with no apparent relation to the structural landmarks of the organ, undergo an acidophilic degeneration and eventually disappear, leaving a pink reticulated mass which rapidly becomes infiltrated with mononuclear and polymorphonuclear cells. If the lesion is near the surface, a cellular exudate extends through and over the surface of the capsule. The cause of the necrosis is not known. Although we have not been able as yet to demonstrate any primary involvement of the vascular supply, the possibility that such a phenomenon may account for the picture must be borne in mind. In addition to the necrotic lesions, the sinuses of the liver contain large numbers of mononuclear cells, many of which are apparently derived from Kupffer cells.

Psittacosis in Monkeys

Lesions in the lungs of parrots, mice, rabbits and guinea-pigs occurred so rarely that we decided to use monkeys in the hope of obtaining an involvement of this organ similar to that described in man. Young *Macacus rhesus* monkeys were employed. At first, intracerebral inoculations of the virus were made. The animals were susceptible to the virus introduced in this manner but showed no changes in the lungs. Then intratracheal and intranasal inoculations were tried, and typical lung lesions were regularly obtained in this manner.

Intratracheal Inoculations.—A number of monkeys were inoculated intratracheally with from 2 to 4 cc. of liver and spleen emulsions from mice infected with psittacosis. All of the animals had high temperatures and roentgenographic evidences of consolidation of the lungs. Some died, and others were killed at different times following inoculation in order to study the various stages of the pathologic picture. A successful subpassage was obtained in one instance, and mice inoculated with an emulsion from this animal's liver died of psittacosis. The lungs at autopsy in all instances were free from ordinary aerobic and anaerobic bacteria. Furthermore, the presence of psittacosis virus in the lungs was demonstrated by inoculation of emulsions of these organs into mice and parrots. Moreover, a monkey inoculated intratracheally with an emulsion of liver and spleen from normal mice developed no fever and no roentgenographic evidence of lung involvement during the period of observation.

Intranasal Inoculation.—Two monkeys were inoculated by intranasal instillation of 1.5 cc. of a liver and spleen emulsion from mice infected with psittacosis. One of the animals developed a high fever two days after inoculation; a roentgenogram revealed marked involvement of the lungs, which was shown at autopsy to be typical of psittacosis.

Roentgen Examinations.—Regardless of whether the virus was administered intratracheally or intranasally, the lesion always began at the hilus and then spread toward the periphery of the lungs. This is excellently demonstrated in the series of roentgenograms of monkey J (fig. 1), which show a striking similarity to those in the preceding paper[2] on psittacosis in the lungs of man.

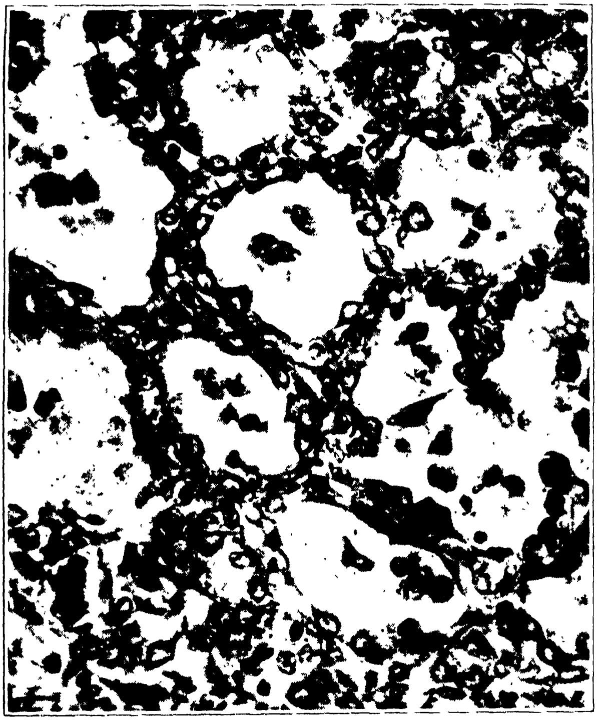

FIG. 4.—A higher magnification of the process shown in figure 3: The alveolar walls are greatly thickened; desquamation of cells into the alveoli is beginning; eosin and methylene blue; slightly reduced from a photomicrograph with a magnification of 450 diameters.

Gross Changes.—Lungs: On opening the thorax, one finds a delicate fibrinous pleurisy over the involved lungs. Areas of consolidation are observed in one or more lobes. Such areas are firm and not very red. On section, their surface is smooth, almost glistening, and mottled with pale zones surrounding the bronchi and blood vessels. No exudate is seen in the bronchi. The consolidation obviously begins at the hilus and spreads toward the periphery of the lung. Hilar lymph nodes are much larger than normal.

Heart: In many of the animals, an increase of pericardial fluid, that may or may not be cloudy, occurs. The heart is usually enlarged, and its muscle is flabby.

Liver: The liver may be enlarged, and at times there is distinct evidence of fatty degeneration. In many of the animals, small areas of necrosis have been observed near the border of the right lobe in the neighborhood of the round ligament.

Spleen: This organ usually appears to be normal.

Microscopic Changes.—Lungs: Examination reveals that the pathologic changes are most intense near the hilus and decrease as the periphery of the organ is approached where some normal alveolar structures may be found (fig. 2). Adjoining the air-containing alveoli is a zone in which the alveolar walls are mildly distended with mononuclear cells and the alveolar cavities contain a moderate number of cells and some precipitated albumin (figs. 3 and 4). In the neighborhood of the hilus, the alveolar walls show ' marked cellular infiltration and the alveolar spaces are filled with fibrin and mononuclear cells (figs. 3 and 5). There is congestion of the capillaries, but the cellular infiltration or proliferation in the alveolar walls is the striking feature (fig. 4). At times the interstitial tissue is so distended by cells that the alveoli are partially or completely obliterated (fig. 5). Moreover, there may be an active proliferation of the alveolar epithelium, giving rise to rows of cuboidal cells lining the alveolar spaces. Occasionally, necrosis is observed. When it occurs, the alveolar walls that are in the necrotic areas evidence hyaline changes. The adventitia of the veins is distended with mononuclear cells, (fig. 3) and the picture resembles the "cuffing of blood vessels" in encephalitic brains. Mononuclear cells—clasmatocytes, plasma cells and lymphocytes—constitute most of the infiltrating elements, but polymorphonuclear cells are observed, particularly in the necrotic areas.

Liver: There is a diffuse fatty degeneration of the liver cells. In the liver of one monkey, areas of focal necrosis, similar to those found in the livers of mice, were observed.

Spleen: A definite increase in the number of large mononuclear cells occurs. In some of the malpighian corpuscles, a central necrosis, associated with hyaline changes in the vessels and depositions of fibrin, is observed.

Etiology

Stained preparations of highly infectious material reveal no ordinary bacteria. Moreover, aerobic and anaerobic cultures of such material have not demonstrated ordinary bacteria. The "minute bodies" described by Levinthal,[5] Lillie[6] and Coles[7] have been found in preparations made from parrots and mice. Such bodies, however, have not been seen in highly infected organs of rabbits and monkeys. Further search for them in these animals should be made. The necessity of this search is emphasized by the fact that infectious organ emulsions from rabbits produced in parrots psittacosis with an abundance of "minute bodies." Furthermore, similar organ emulsions from mon-

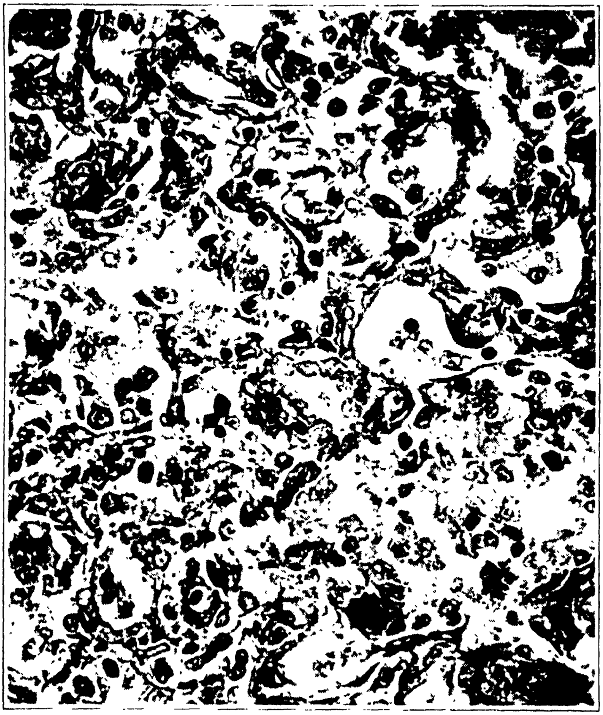

FIG. 5.—An advanced stage of the process shown in figure 4: The alveoli are either filled with mononuclear cells or collapsed by the great thickening of the alveolar walls; eosin and methylene blue; slightly reduced from a photomicrograph with a magnification of 450 diameters.

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5. Levinthal, W.: Klin. Wchnschr. 9: 654, 1930.
6. Lillie, R. D.: Pub. Health Rept. 45: 773 (April 11) 1930.
7. Coles, A. C.: Lancet 1: 1011 (May 10) 1930.

keys, in which these bodies were not seen, induced in mice psittacosis associated with the presence of the small bacteria-like structures. The nature of these small structures and their relation to psittacosis are not known.

SUMMARY

1. The virus of psittacosis is in the feces and in the material collected from the nose, mouth and procrop of infected parrots.
2. Parrots and monkeys can be infected by intranasal instillations of the virus.
3. Parrots and rabbits that have recovered from a primary infection are refractory to reinfection.
4. It is not a simple matter to demonstrate neutralizing properties in convalescent human serum.
5. In parrots and in mice, the principal lesions occur in the liver and spleen.
6. Young monkeys (*Macacus rhesus*) are susceptible to intracerebral, intratracheal and intranasal inoculations of psittacosis virus. When it is instilled in the nose or injected in the trachea a characteristic pathologic picture occurs in the lungs which is similar to that observed in man.

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CULTIVATION OF VACCINE VIRUS

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(Received for publication, June 21, 1930)

Many investigators have cultivated vaccine virus in bits of living tissue embedded in plasma. These small cultures were made on cover-slips which were then inverted and sealed over hollow ground slides. For many reasons methods of making larger cultures are highly desirable. To this end, Carrel and Rivers (1) devised a procedure whereby cultures of vaccine virus were made in 2 to 8 cc. amounts. This was accomplished by inoculating minced chick embryo tissue with vaccine virus and then embedding it in diluted chicken plasma in Carrel flasks. This method was improved upon by Maitland and Maitland (2) who used a fluid medium consisting of minced chicken kidney suspended in chicken serum diluted with Tyrode's solution. Later Muckenfuss and Rivers (3) altered Maitland's medium by substituting rabbit testis and serum for chicken kidney and serum. The fact that certain viruses, *e.g.*, vaccine virus, Virus III, herpetic virus, will multiply in a medium of this nature has been amply verified by the work of Eagles and McClean (4), Andrewes (5–7), and Maitland and Laing (8).

The work to be described at this time has to do with the simplification of the technique for the cultivation of vaccine virus suitable for Jennerian prophylaxis in man. A medium, in which tissues and serum from halfgrown or adult animals are used, may easily permit the entry of an unknown virus into the cultures as a contaminant. The chance of such an occurrence can be lessened by the use of minced chick embryo suspended in Tyrode's solution. The possibility that such a medium would be appropriate was suggested by an experiment in which Andrewes (6) showed that Virus III survived and formed inclusions in rabbit testicular tissue suspended in Tyrode's solution.

Methods and Materials

Virus.—Levaditi neurovaccine virus that had been propagated for 6 months in rabbit testicles was used to initiate the cultures.

Tissues.—Testicular tissue was obtained aseptically from rabbits, washed in Tyrode's solution, placed in a sterile watchglass contained in a Petri dish, minced with scissors, and then distributed in proper amounts into flasks by means of a pipette. Embryo tissue was obtained from eggs, incubated 9 to 12 days, that had been opened according to the method of Carrel and Rivers (1) or that of Eagles and McClean (4).

Fluids.—When serum was used it was collected from rabbits, and diluted with 3 parts of Tyrode's solution. Tyrode's solution, pH about 7.2, prepared according to the following formula was sterilized by filtration: NaCl, 8 gm.; KCl, 0.2 gm.; $CaCl_2$, 0.2 gm.; $MgCl_2$, 0.1 gm.; $NaHPO_4$, 0.05 gm.; $NaHCO_3$, 1.0 gm.; glucose, 1.0 gm.; water *q.s.* 1000 cc.

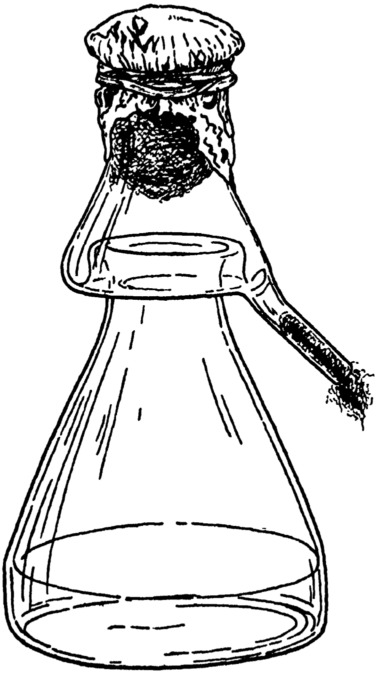
Containers.—Containers for the production of vaccine virus in large amounts must be of sufficient size and easy to handle. Furthermore, they must permit aeration and prevent contaminations and evaporation. At first, cultures were made in Carrel D flasks. Then 50 cc. Erlenmeyer flasks, stoppered with cotton over which two layers of tinfoil were placed, were used. Neither type of container was found entirely satisfactory for the work. Finally a flask was designed (collar flask) which proved to be suitable. The mouth of the flask (Text-fig. 1) is of sufficient size to permit the removal of the contents by means of a pipette. It is closed by a cotton plug over which are stretched two or three layers of tinfoil. The enlargement in the neck reduces the amount of water of condensation on or near the cotton plug and also prevents the water that does touch the cotton from running down and contaminating the culture. The small opening, plugged with cotton, on the side serves for ventilation. The bottom is perfectly flat, thus providing a large area over which a thin layer of culture may be evenly distributed. The flasks are made in two sizes. The dimensions of the large one are: height, 14 cm., diameter of mouth, 3.5 cm., diameter of base, 9 cm. The measurements of the small one are: height, 9 cm., diameter of mouth, 2.5 cm., diameter of base, 4.5 cm. In the large flasks, 2 to 4 gm. of tissue suspended in 15 cc. of Tyrode's solution were used, while in the small ones 0.5 to 1 gm. of tissue suspended in 5 cc. of Tyrode's solution was employed.

Preparation of Cultures.—Approximately 1 gm. of tissue was used to each 5 cc. of diluted serum or Tyrode's solution. Testicular or chick embryo tissue suspended in its vehicle was distributed in flasks. Prior to use, the sterility of the medium was tested on blood agar and in broth. During this interval (48 hours), the prepared flasks were stored at +5°C. The medium was then inoculated with 0.1 to 0.2 cc. of virus emulsion, and the cultures were incubated at 37°C. for 7 days. New cultures were made by the direct transfer of a few drops of the old culture into flasks of fresh medium. In this simple manner, vaccine virus can be propagated through an indefinite number of culture generations.

Titration of Virus.—Cultures for titration were ground in sterile mortars, and then appropriate dilutions were made with Locke's solution. A fresh pipette was always used for each successive dilution. 0.25 cc. of each dilution was injected into the shaved skin of rabbits. Daily observations of the animals were made for a week.

EXPERIMENTAL

In our investigation concerning the cultivation of vaccine virus, many experiments have been performed. All of them will not be described, inasmuch as some are similar to experiments made and reported by others (8). Part of our work, however, is different from that previously described and the results are summarized in Chart 1.



TEXT-FIG. 1. Type of flask used for the cultivation of vaccine virus

The first cultures of Series A (Chart 1) were made February 17, 1930. A medium consisting of rabbit testicular tissue and a mixture of rabbit serum (1 part) and Tyrode's solution (3 parts) was inoculated with vaccine virus and then distributed in Carrel D flasks. Virus from a culture of the third generation of Series A was inoculated into a flask (Series B) of medium comprised of chick embryo tissue suspended in rabbit serum (1 part) and Tyrode's solution (3 parts). Seventh generation virus (Series B) was inoculated into a medium of chick embryo tissue suspended in Tyrode's solution (Series C). At this time collar flasks were first employed and since then they have been in general use.

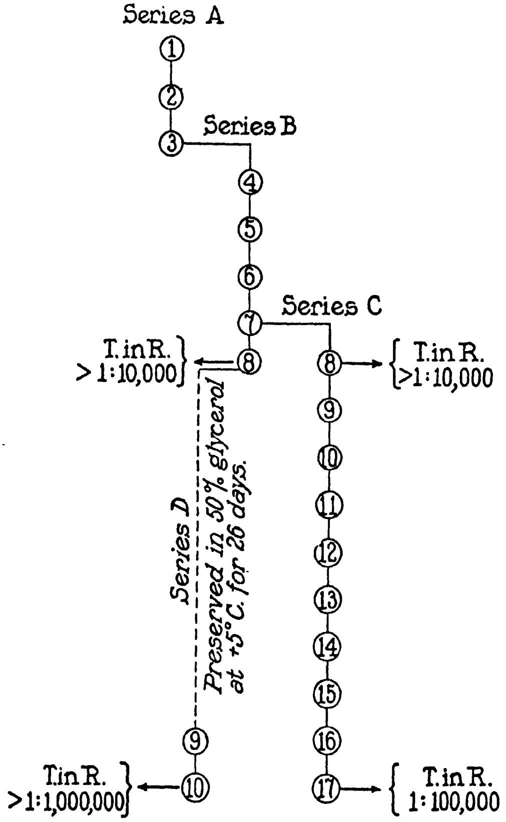


CHART 1. Summary of experiments on cultivation of vaccine virus.

The media used in the different series were as follows: Series A: Rabbit testicular tissue in a mixture of rabbit serum and Tyrode's solution. Series B: Chick embryo tissue in a mixture of rabbit serum and Tyrode's solution. Series C and D: Chick embryo tissue in Tyrode's solution.

T. in R. indicates titer in rabbit.

The virus in Series C has been propagated for 10 generations, and is still under cultivation.

The fact that the virus multiplied is obvious and a mathematical proof seems superfluous. Nevertheless, one estimation will be given. The titer of the eighth generation virus (Series B) was >1:10,000. This virus mixed with glycerol was stored at +5°C. for 26 days. Then the preserved virus was diluted 1:100 with Tyrode's solution, and 5 drops of the dilution were inoculated into 5 cc. of fresh medium. After a 9-day period of incubation, 4 drops of this culture were added to 15 cc. of fresh medium. After incubation the titer of the last culture was 1:1,000,000 (Series D).

From the work summarized in Chart 1, it is obvious that the virus can be transferred without difficulty from one kind of medium to another. Furthermore, culture virus, mixed with glycerol, sealed, and stored at +5°C., can be preserved for a long time. In this manner virus has been preserved for 64 days without great loss in potency. Moreover, the preserved virus can be employed as an inoculum for the initiation of fresh cultures.

The virus propagated in cultures for a long time seems to have lost none of its characteristics, for, when it is spread on the scarified shaved skin of a rabbit, typical vaccinal lesions occur. Moreover, serum from a rabbit immune to Levaditi's neurovaccine virus completely neutralizes the culture virus. Finally, an immune serum prepared with the culture virus neutralizes the Levaditi vaccine virus.

DISCUSSION

A simple method for the *in vitro* cultivation of vaccine virus has been devised. The flasks employed are easy to manipulate and permit the production of large amounts of virus. Moreover, the medium is innocuous and inexpensive, consisting of minced chick embryo suspended in Tyrode's solution. In spite of the simplicity of the procedure, however, one should not forget the fact that living susceptible host cells are employed. No one has as yet been able to demonstrate that vaccine virus can multiply in the absence of such cells.

The facility with which vaccine virus can be handled and cultivated *in vitro* varies with the strain. This fact has been evidenced by previous experiences in our laboratory and also in that of Eagles and

McClean (4). In the work reported at this time a neurovaccine virus was employed. Studies have been planned to determine whether it is possible to adapt a dermovirus to the type of medium used by us.

SUMMARY

1. A strain of neurovaccine virus was cultivated in a medium consisting of minced chick embryo suspended in Tyrode's solution.
2. The virus upon cultivation apparently lost none of its essential characteristics.
3. The culture virus can be preserved and stored for long periods of time. Furthermore, the preserved virus can be used to initiate fresh cultures.

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OBSERVATIONS ON THE COURSES OF DIFFERENT TYPES OF BRIGHT'S DISEASE, AND ON THE RESULTANT CHANGES IN RENAL ANATOMY

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[1] The clinic was organized by Dr. Stillman, and with him the general plan of the problem was worked out in its first years. Dr. Møller carried part of the clinical responsibilities during the years 1926–28, and performed the chief part of the difficult task of sifting, ordering, selecting, and charting the accumulated data of former years. Dr. Ehrich has contributed the descriptions of the anatomical changes found in the kidneys at autopsy. Dr. McIntosh carried for five years part of the clinical responsibilities, and assisted in final preparation of the material for publication. Drs. Leiter and MacKay were each associated with the clinic for two years, and Drs. Hannon, Moore, and Johnston for one year. Dr. Moore also assisted in preparing the charts, and Dr. Hannon the text, in the forms finally published.—D. D. V. S.

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The study of nephritis beginning with Bright (1836) has led to the recognition of different types of renal disease which have finally received consistent outlines in the classic monographs of Volhard and Fahr (1914) and Volhard (1918), and the recent work of Addis (1925, 1928). These authors, approaching the problem from considerably different directions, nevertheless agree in the main, and recognize three chief types of nephropathy, of which the outstanding differential characteristics are: (1) Hematuria, acute, intermittent, or chronic, usually with hypertension, and with nitrogen retention, frequent in the acute stage, regular in the advanced chronic stages; (2) marked hypertension, which precedes any serious renal signs; (3) edema and heavy proteinuria without hematuria or hypertension. These three conditions are characterized by Volhard and Fahr as glomerulonephritis, nephrosclerosis ("Nierensklerose"), and nephrosis, respectively; while Addis calls them hemorrhagic, arteriosclerotic. and degenerative Bright's disease.

It was the great service of Volhard and Fahr not only to introduce order and clarity into the clinical classification of nephritides, but also for the first time satisfactorily to relate the clinically defined conditions to anatomical changes in the kidneys. The outstanding and primary histological changes which they observed in the three conditions were respectively: (1) Inflammatory glomerular destruction; (2) thickening of the small renal arteries; and (3) degeneration affecting most markedly the tubules. These were the primary and characteristic changes noted, although the autopsy material yielded by terminal conditions frequently presented in varying degree additional secondary changes. Subsequent observations, including those presented in this paper, have confirmed the essentials, clinical and histological, of Volhard and Fahr's classification.

In this report we present, from a series of cases observed closely over periods varying from weeks to years, objective functional and clinical data, together, in those cases which have come to autopsy, with descriptions of the terminal histology. The data are presented with the hope of adding to the clarity and completeness with which the variable courses of these different types of renal disease can be pictured, and thereby lessening the avoidable error of diagnosis and prognosis.

The object of this contribution is limited by the boundaries of the above statement. Etiology and therapy have been kept constantly in view during the progress of the work, but will enter only incidentally into this presentation.

Nomenclature

With regard to the last two of the three types of renal disease mentioned, there appears to be little difference between the Volhard-Fahr and Addis nomenclatures. "Nephrosclerosis" and "arteriosclerotic Bright's disease" are so nearly equivalent, both in their apparent significance and in the meaning attached to them, that they may be regarded as synonymous, and we have used them as such.

Similarly both Volhard and Fahr, and Addis, speak of the "degenerative" renal diseases. However, the former authors also use nephrosis, as a convenient and familiar synonym; while Addis, in view apparently of confusion that has grown up about the term from controversies concerning the nature of the disease, has abandoned "nephrosis" and uses only "degenerative Bright's disease." We have had somewhat the same feeling, and have to a large extent used the term, "degenerative Bright's disease." We have employed the shorter synonym, however, when the repeated use of the longer one would lead to awkwardness of expression. Doubtless, as Volhard remarks in the introduction to his later monograph (1918), it is more essential to attain clarity concerning the nature of the disease than to be meticulous concerning the etymology of its name.

In the case of the hemorrhagic or glomerular type although there appears to be no difference between Addis and Volhard and Fahr concerning the identity of the disease, there is a difference in the manner in which they have divided it into stages and sub-types. Volhard and Fahr divide the disease into the diffuse and focal forms of glomerulonephritis, which they differentiate chiefly by means of blood pressure, the focal ("herdförmige") nephritis being distinguished by the fact that, from its acute hemorrhagic onset, throughout its course, acute or chronic, the blood pressure remains normal. Likewise Volhard and Fahr appear to consider maintenance of renal function as a characteristic of this type of the disease, except in complicated cases. We have not found it easy to differentiate our cases on the basis of blood pressure, without inconsistency with regard to the renal function.

TABLE 1

TYPE OF DISEASE		CLINICAL COURSE	URINE SEDIMENT (ADDIS)	ANATOMICAL CHANGES OF KIDNEYS
Hemorrhagic (glomerulonephritis)		Acute onset. Either heals, improves to a latent condition, or progresses through an intermediate chronic state, usually edematous, with diminishing renal function, to terminate in uremia	Red cells in varying numbers. Blood, epithelial, and granular casts, in all stages except terminal. Hyaline casts in all stages. Broad "renal failure" casts in terminal	*Glomerular inflammation* leading in terminal stage to nearly complete destruction. Intracapillary and extracapillary forms of Fahr. Also varying tubular degeneration and arterial changes
Non-hemorrhagic	Arteriosclerotic (nephrosclerosis)	Insidious onset. Marked hypertension. No edema unless cardiac. Death by cardiac failure, apoplexy or uremia	Chiefly hyaline casts	*Arterioles are diseased*, with contracted lumina, endarteritis, intimal hyperplasia, fatty degeneration, necrosis, in varying degree. Varying proportions of glomeruli destroyed. No marked tubular degeneration. Necrosis of tubules may occur
	Degenerative (nephrosis, lipoid or amyloid)	Insidious onset. Edema, and proteinuria. No hypertension. May end in cure, death by intercurrent infection, or less frequently uremia	Chiefly hyaline casts, few epithelial, fatty, granular, and waxy. No blood casts. Failure casts in cases with terminal uremia. Doubly refracting globules	*Degenerated tubular epithelium.* Varying proportions of glomeruli may be destroyed; hyaline or amyloid. In amyloid type arteriolar walls are more or less infiltrated by amyloid material

Thus cases 10 and 11 showed decreases of renal function, measured by the blood urea clearance, to 12 and 4 per cent of normal respectively, at minimum points reached 6 and 3 weeks respectively after hemorrhagic onset: yet neither at any time under observation showed hypertension. Case 18 progressed steadily downwards from the acute through the chronic stage, and is now apparently about to enter the terminal, but has at no time shown hypertension. It has been difficult to conceive that these cases in their renal disease are essentially different from the others in which hypertension, usually transitory, occurred during the first weeks of the acute period.

Addis does not, in his outline of the course of hemorrhagic nephritis, attempt to make a clinical separation of the focal cases. Furthermore his description of the progress of the disease, from the acute onset upwards to cure or to arrest in the latent stage, or downwards through the active chronic to the terminal stage, so precisely fits our observations, that in describing the clinical progress of our cases the use of his terms has been almost unavoidable. We have accordingly used Addis's terminology in the discussion of our observations concerning the course of "hemorrhagic nephritis."

In the discussion of the pathological histology, however, Fahr's anatomical terminology (Volhard and Fahr, 1914) has been followed. One might expect confusion to arise from using one set of terms to indicate conditions in the living cases and another set to outline the anatomical post mortem findings. In fact, however, there appears to be no such confusion: the use of one term to indicate the clinically observed condition and another to express post mortem observations serves the purpose of indicating whether the renal condition under consideration is being discussed on the basis of clinical or anatomical data.

In a semi-diagrammatic and incomplete, condensed form, the more outstanding clinical characteristics of the three main types of renal disease may be represented in table 1 (the synonyms of Volhard and Fahr are enclosed in parentheses).

Details concerning the occurrence of these and other characteristics in each type of renal disease will develop later in the discussion of specific observations covering a group of cases of each type.

CASES PRESENTED

The 67 cases presented have been selected for the most part because they are the ones we have had opportunity to observe most completely. In addition a few that were under observation for but short periods before death are included for the reason that we obtained autopsies on them.

The cases are grouped as follows with regard to the clinical types of renal disease which they represent and their outcomes. In each group the cases with most favorable outcome, or in which the course of the disease has appeared to be least rapid or severe, have been placed first, while the cases with the most unfavorable or most rapidly fatal outcome have been placed last.

Acute hemorrhagic nephritis. Nos. 1 to 23.

Nos. 1 to 5 inclusive recovered.

Nos. 6 to 16 improved to the latent stage.

Nos. 17 and 18 progressed to the active chronic stage, but still live.

Nos. 19 to 23 progressed to the terminal stage and died in from 3 months to 4 years after the initial acute onset.

Cases admitted in latent form of hemorrhagic nephritis. Nos. 23a and 24.

Both these cases progressed into the terminal condition, one after 4 years and the other after 8 years, in the latent stage. Case 23a still lives but case 24 has died in uremia.

Cases admitted in the active chronic form of hemorrhagic nephritis. Nos. 25 to 37.

Nos. 25 to 30 are progressing but still alive, from 1.5 to 6 years after the first symptoms of their disease appeared.

Nos. 31 to 37 died 2.5 to 12 years after onset of disease.

Terminal stage of hemorrhagic nephritis. Nos. 38 to 50.

All the patients who were in the terminal stage when admitted have died, at periods from 6 months to 13 years after symptoms of nephritis were noticed. In the cases of longest duration there had apparently been latent periods.

Arteriosclerotic nephritis. Nos. 51 to 56.

These patients have all died at periods varying from 3 to 10 years after hypertension was noted. One death appeared to be due to cardiac failure, two were typically uremic, while in the other 3 symptoms of uremia and heart failure were so combined that it was difficult to ascribe exitus more to one than to the other.

Degenerative nephritis or nephrosis. Nos. 57 to 66.

No. 57 made an apparently complete recovery.

Of nos. 58 to 64, 4 are still living, with the disease in an apparently chronic form, and 3 have died from causes other than renal failure.

Nos. 65 and 66 died in typical uremia with nitrogen retention. Of these no. 66 at autopsy was found to be amyloid: autopsy on no. 65 was not permitted.

Significance of Clinical Data Presented

In order to present within practicable space observations on so large a series of patients over prolonged periods it is necessary to use graphic methods and limit the data to those of most definite significance. We have accordingly given the results of one functional test, the blood urea clearance, which has proved the most sensitive and reliable. The plasma protein contents have been given as the figures most closely related to the edematous tendency and to the progress of the degenerative type of Bright's disease, hemorrhagic or non-hemorrhagic. The edema itself is represented in a semi-quantitative way, and likewise the proteinuria that, in gross form, is part of the degenerative syndrome. Blood pressure values are given because of their aid in differentiating arteriosclerotic nephritis from the other types, and of their interest in showing something of the incidence of circulatory derangement in hemorrhagic nephritis. Hematuria is shown because of its diagnostic significance in differentiating hemorrhagic from pure degenerative and arteriosclerotic nephritides. Hemoglobin concentrations in the blood are presented because anemia appears to represent one effect of nephritic toxic injury which is measurable, even though it is less serious for the organism than those injuries directly responsible for the uremic syndrome.

The blood urea clearances were determined as described by Møller, McIntosh, and Van Slyke (1928). Each clearance involves urea determinations on one blood sample and on two urine samples passed during successive periods of approximately one hour each.

The plasma proteins were determined by the method of Howe (1921), the globulins being precipitated by 22 per cent sodium sulfate and the albumin determined in the filtrate, all analyses being made by micro Kjeldahl methods.

Hemoglobin concentrations in blood were determined by the method of Palmer (1918).

Proteins in the urine have been determined in a majority of the cases presented by the approximate Esbach method. During the past three years the more nearly quantitative procedure of Shevky and Stafford (1923) has been employed. Because of the semi-quantitative nature of most of the urine protein values we have plotted them in a semi-quantitative manner in the charts.

Hematuria in most of the cases, unless macroscopic, has been determined by the usual microscopic examination of sediment obtained from morning urine. During the past 3 years the quantitative sediment count of Addis (1926), on sediment obtained from urine passed during the last 12 hours of a 24-hour dry diet, has been introduced. Some of the results obtained with it are given in the case histories. Because it was not used in most of the cases presented, we have not designed the charts to indicate millions of cells excreted, but only the relative hematuria roughly differentiated into 4 degrees (see explanation before the charts of the hemorrhagic cases).

Blood pressures have been measured with mercury manometers.

The significances of the above values are discussed below in more detail. The discussion is based in part on the observations of other authors, in part on records presented later in this paper.

Blood Urea Clearance

The blood urea clearance is used in our charts as the measure of renal functional ability. That urea retention is characteristic of renal failure leading to clinical uremia has long been recognized. Van Slyke, McIntosh, Møller, Hannon, and Johnston (1930) have presented data showing that the urea excreting ability of the kidneys was, in fact, the most sensitive indicator of the state of the renal function of several which they tested, including blood urea and blood creatinine concentrations, and phenosulfonephthalein excretion. As a measure of the urea excreting power of the kidneys it has been recognized since the work of Ambard (1914) and F. C. McLean (1915, 1917) that the most exact information requires comparison of both blood urea concentration and urea excretion in the urine. From the work of Møller, McIntosh, and Van Slyke (1928), it appeared that the simplest and most satisfactory way to express the relationship between these two factors was by means of the "blood urea clearance," by which term they indicated the *cubic centimeters of blood per minute cleared of urea by renal excretion.*

For urine volumes below 2 cc. per minute, as shown previously by Austin, Stillman, and Van Slyke (1921) the blood urea clearance decreases with diminishing urine volume. Diminishing the volume to 1/4 was found to lower the clearance to 1/2: in other words, the clearance varies in proportion to the square root of the urine volume. Making allowance for this influence of urine volume, the blood urea clearance, from determinations with any urine volume below 2 cc. per minute, is estimated for the standard condition that urine volume output is 1 cc. per minute (approximately the normal average output), the formula (Møller, McIntosh, and Van Slyke, 1928) used being:

$$\text{Standard blood urea clearance} = \frac{U}{B}\sqrt{V}$$

where U is the concentration of urea in the urine, B is the concentration in the blood, and V is the volume output of urine in cc. per minute. Van Slyke, McIntosh, Møller, Hannon, and Johnston (1930) found that the relative sensitiveness to renal damage of this clearance was such that it indicated a drop below 40 per cent of normal renal function in a large percentage of progressing nephritic cases when phenosulfonephthalein excretion, blood creatinine, and blood urea considered without its relation to urea excretion, were still within the range of normal variation; and that, in point of time, the blood urea clearance in progressing cases revealed development of functional deficit weeks or months before the other tests above mentioned. Because of the sensitiveness and practicability of the blood urea clearance as a measure of renal function, we have used this one value for the purpose of expressing renal efficiency. In the charts the standard clearance is indicated by the symbol C_s, and is expressed both in cubic centimeters of blood cleared per minute, and in percentages of the average normal clearance, which is 54 cc. per minute for adults. Correction has been made for body size according to the chart for this purpose published by McIntosh, Møller, and Van Slyke (1928), although such correction is important only for children.

For urine volumes above 2 cc. per minute Austin, Stillman, and Van Slyke (1921) showed that the clearance is independent of the volume. With urine volumes over 2 cc. per minute the clearance is calculated simply as $\frac{UV}{B}$. The average value of this "maximum clearance" for normal adults is 75 cc. of blood cleared of urea per minute. Møller, McIntosh, and Van Slyke (1928) in studies on nephritic patients found that with a given subject the same percentages of average normal function are given by the "maximum" blood urea clearance, determined with high urine volume outputs, as by the "standard" blood urea clearance, determined with urine outputs within the usual range. In cases where during the clearance determinations the urine volumes exceeded 2 cc. per minute, we have calculated the maximum clearance in percentage of normal and recorded it as such in the charts. Since urine volumes in hospital patients seldom exceed 2 cc. per minute, unless diuresis is intentionally stimulated, however, nearly all the clearance values shown are "standard clearances."

From a comparison of the terminal clearance values and autopsy findings in cases reported in this paper it appears that in *hemorrhagic and degenerative nephritis one may interpret the blood urea clearance as a measure of the proportion of glomerular tissue still functioning.* In terminal arteriosclerotic nephritis, clearance values which are a small fraction of normal have been found with a large proportion of glomeruli still intact. *It appears probable that in arteriosclerotic nephritis the fall in blood urea clearance is proportional to the decrease in renal blood flow rather than to the glomerular destruction.*

Plasma Protein Content and Edema

As urea retention in nephritis is the sign of a condition leading to uremia, so is plasma albumin deficit the sign of a condition leading to edema. As, after renal failure, edema is the complication that causes most frequent concern, so is determination of the plasma proteins, after that of the urea excreting ability, the quantitative chemical examination to which we have come to refer most frequently in judging the condition of patients.

In a recent report on results from 75 nephritic patients Moore and Van Slyke (1930) have shown that when the total protein content, normally averaging 7 per cent, falls below 5.2 to 5.8 per cent, or the albumin, normally averaging 4.3 per cent, falls below 2.3 to 2.7 per cent, or the plasma specific gravity, normally averaging 1.027 falls below 1.0225 to 1.0235, edema is usually present. The figure most closely connected with the edematous tendency appears to be the albumin, but as the globulin usually remains unaffected, the total proteins and the specific gravity, which reflects the total proteins, as a rule both parallel the albumin and show the same correlation with edema.

While urea retention is a warning, and not in itself apparently the cause of uremia, there is fair proof that plasma protein deficit is an important direct cause of non-cardiac nephritic edema.

Evidence that plasma protein deficit predisposes to edema was first given by the physiological experiments of Starling (1895–96). He measured the osmotic pressure of the plasma proteins and found it at a level of about 30 mm., between arterial and venous blood pressures. Starling pointed out that it is presumably this osmotic attraction of the plasma proteins for water that balances in the capillaries the hydraulic pressure tending to force the fluid out into the tissue spaces, and that when the protein osmotic pressure weakens, because of decrease in protein concentration, undue amounts of fluid are likely to pass out into the tissues. He found that the edematous leg of a dog perfused with Ringer's solution remained edematous, but when perfused with serum the edema fluid was reabsorbed. The salts present, although in molar concentration many times exceeding the proteins, have relatively little effect in controlling fluid diffusion because the salts themselves diffuse freely through the capillary walls.

That proteins are scant in the plasma of many nephritic patients was noted by Bright (1836). Csatary (1891) noted that the deficiency affected the serum albumin more than it did the globulin, so that the albumin: globulin ratio, normally 1.5 to 2.0, frequently fell below 1 in nephritis. These observations have been confirmed and amplified by other authors, whose work has been reviewed by Linder, Lundsgaard, and Van Slyke (1924). It was Epstein (1917), however, who connected the observation of plasma protein deficit with Starling's experimental and theoretical work to form an explanation of the cause of non-cardiac edema in nephritis. This explanation, viz., that the decreased osmotic attraction of the proteins for water permits the escape of the latter into the tissues, has been confirmed by the work of Govaerts (1924), of Schade and Claussen (1924) and of Cope (1928) who determined directly the osmotic pressure in the sera of normal subjects and of nephritic patients with and without edema.

As might be expected, the relation between protein deficit and edema formation is not an entirely regular and uniform one. There are other factors which resist or reënforce the hydropigenous effect of plasma protein deficit. With plasma proteins near the level at which their deficit usually produces edema, the latter may be present or not, and may come and go in the same subject, as these other modifying factors exert their influence in one direction or the other. Salt intake is such an influence: a patient with fairly low plasma protein content and edema may lose the latter merely by being put on a salt free régime, although, as Moore and Van Slyke (1930) have shown, entire disappearance of edema is infrequent if the proteins are below the above quoted critical levels. There are other less tangible influences. In a patient with tendency to edema barely under control edema may appear after infections or operations, and disappear during recovery. In some instances fever appears to tend to make edema disappear; in others vomiting shows a desiccating effect. And in some cases on the border line edema comes and goes for no observable reason.

In the first weeks of acute nephritis edema may occur with plasma protein level normal; this edema is due to some influence quite apart from the osmotic effect of the plasma proteins; it may arise from some toxic effect increasing capillary permeability. Again in the terminal stage of hemorrhagic or arteriosclerotic nephritis edema may occur as the result of heart failure.

With these exceptions, the accumulated data indicate that the constant and dominating factor in producing non-cardiac edema in Bright's disease is plasma albumin deficit, the effect of which is only modified in degree by other influences, and that Epstein's application of Starling's theory has been justified by the studies of subsequent investigators. The data presented in the charts of this monograph provide further support for the above statement, at the same time that they furnish examples of the effects of other influences in modifying that of plasma protein deficit.

The fact that tendency to edema formation is closely related to plasma albumin deficit and relatively unaffected by globulin changes is explained by Govaerts' (1924) finding that the albumin exerts four times as much osmotic pressure per gram as the globulin.

Proteinuria

Since proteinuria was shown by Bright to accompany renal disease it has maintained its place as a sign of primary diagnostic importance. To its original significance as an evidence of renal injury recent years have added another. Heavy protein excretion in the urine is accompanied in almost all cases by protein deficit in the blood plasma, and there is a rough parallelism between the two. As indicated by the observations of Linder, Lundsgaard, and Van Slyke (1924), loss of albumin in the urine does not appear to be the sole cause of albumin deficit in the blood plasma. The malnutrition common in the edematous types of nephritis adds its effect, apparently by retarding regeneration of albumin. In a rough approximate way, however, the magnitude of protein excretion corresponds with the degree of protein deficit in the blood plasma. Both gross proteinuria and plasma protein deficit are the almost regular accompaniments of the degenerative types of renal disease, hemorrhagic and non-hemorrhagic. The protein that appears in the urine in these types of nephritis (except the amyloid) is 85 per cent or more albumin, relatively little globulin being excreted in the urine or missing from the plasma (for data and literature see Hiller, McIntosh, and Van Slyke, 1927). Correspondingly, as mentioned above, the protein loss in the plasma is as a rule confined to the albumin fraction.

It is in the degenerative types that the urinary protein output has the greatest interest, but in all types it assists in the diagnosis, the scanty output in the sclerotic type helping with the hypertension to differentiate the condition from the degenerative and hemorrhagic, or, in the terms of Volhard and Fahr (1914), from the nephrotic and glomerular types of renal disease.

Hematuria

Gross hematuria has long been recognized as a common sign of acute, and microscopic hematuria as one of chronic, glomerular nephritis (Volhard and Fahr, 1914). Addis (1925) points out that of all the clinically observable abnormalities of glomerular nephritis, hematuria determined under standardized conditions, is the one which is most characteristic and of most assistance in differentiating the condition from the pure degenerative (nephrosis) and arteriosclerotic types. In order to obtain as nearly as possible a quantitative measure of hematuria Addis puts his patients for 24 hours on a dry diet and centrifugates the urine passed during the last 12 hours. Thus a concentrated urine is usually obtained in which the red cells do not dissolve. The latter are counted in the sediment. In part of our cases Addis' sediment counts have been made and are recorded in the case histories. Unfortunately most of the observations reported in this paper were made before Addis' procedure was introduced into our clinic. The results shown, even by the less precise ordinary microscopic procedure, however, go far to confirm the importance that Addis attaches to hematuria as a diagnostic sign of hemorrhagic or glomerular nephritis. In the acute and terminal stages of the disease hematuria, macroscopic or readily detectible by ordinary microscopic observation, has been constant. During the active chronic stage hematuria has usually been constant, sometimes intermittent, as determined by ordinary examination. Since the introduction of the Addis sediment count microscopic hematuria in excess of normal has been found to be practically constant in the active chronic stage. The one phase of the "hemorrhagic" disease which is not hemorrhagic is encountered during improvement or recovery from the acute disease. The hematuria may completely disappear while there is still heavy proteinuria, albumin deficit in the plasma, and some tendency to edema. There follows then a period of some weeks or months during which such a case is indistinguishable, except for its history, from a pure nephrosis. With this exception it appears that some degree of hematuria is nearly constant throughout all stages of the condition which Volhard and Fahr call glomerular nephritis and Addis terms hemorrhagic Bright's disease. That hematuria may also occur in the terminal collapse of what Volhard and Fahr term the malignant type of nephrosclerosis does not detract seriously from its value as a diagnostic characteristic.

Anemia

Brown and Roth (1922) have reviewed the literature concerning anemia in nephritis, and have presented data indicating that the anemia is due to injury of the bone marrow, which tissue shares in the constitutional damage that is known to be suffered by the heart, retina, and blood vessels. Brown and Roth (1922, 1923) further presented figures showing a definite prognostic significance of anemia in nephritis. In a group of 139 cases, those with no anemia showed in 2.5 years 18 per cent mortality. Those with 60 to 85 per cent of normal hemoglobin content showed 46 per cent, and those with less than 60 per cent of normal hemoglobin showed 85 per cent.

Blood Pressure

Hypertension is the quantitatively measurable effect of circulatory change in nephritis. The literature up to 1913 has been summarized by Janeway (1912–13). The conclusions concerning the relation of hypertension to the different types of renal disease reached from his own observation by Janeway can still be quoted unamended. They are as follows. We have changed only the order, and prefixed to the conclusion concerning each condition the name given that condition in the terminology now used.

1. *Hemorrhagic nephritis* (*glomerular nephriis*). "Hypertension may arise in connection with the unknown intoxication which causes disturbances in the central nervous system and which we call uremia. . . . . Clinically this intoxication is associated with severe acute nephritis, sometimes at its very onset, besides the subacute and chronic inflammatory affections of the kidney."
2. *Arteriosclerotic nephritis* (*nephrosclerosis*). "Hypertension may arise in primary irritability of the vasoconstricting mechanism from unknown, probably extrarenal, causes, which lead eventually to arteriolar sclerosis. In this type the disease of the kidney is the sequence, not the cause, of the generalized vascular lesion. When it progresses to a condition of extreme atrophy, resulting in the true primary contracted kidney, a renal element may be added to the existing hypertension."

According to Janeway's conception the hypertension of hemorrhagic nephritis is a result of the renal disease, while that of arteriosclerotic type precedes the renal disease. In this conception Volhard and Fahr (1914) concur. Branch and Linder (1926) in autopsies of 7 cases of typical terminal hemorrhagic nephritic deaths, preceded by periods of nitrogen retention and hypertension, found in one case no arterial changes. Their findings support Janeway's conclusions.

Summary of Chief Significances of Data Charted

The apparent significance of the different values we have charted may be summarized as follows.

Blood urea clearance. Measure of intact glomeruli in hemorrhagic and degenerative cases, of renal circulation in arteriosclerotic cases.

Hematuria. Sign of active inflammation in glomeruli.

Hypertension. Sign of circulatory changes. When high and prolonged indicates anatomical changes in small arteries.

Plasma protein deficit. *Gross proteinuria.* *Edema.*	Signs of degenerative syndrome. Accompany degenerative renal changes, with or without glomerular damage.

Anemia. One of the indications of toxic damage to organism.

Edema, besides accompanying the degenerative syndrome, also occurs, as mentioned above, in the initial weeks of acute hemorrhagic nephritis, and in the cardiac failure that frequently forms part of the terminal failure.

Uncharted Data

Besides the data above listed, and recorded on the graphic charts, we have, either continuously or at stages during the ten years which these studies cover, made observations of most of the other signs and functional measurements which, according to the current literature, have given promise of throwing light on the conditions of the patients.

As measures of renal function the concentration and dilution test was carried on for several years, and the phenolsulfonephthalein excretion test of Rowntree and Geraghty (1910) has been carried out parallel with the blood urea clearance throughout the course of these studies. The concentration and dilution test is so subject to the influence of water retention and elimination by edematous patients, and to the influence of variations in the salt content of the diet, that it is less reliable than the blood urea clearance. The phthalein excretion, as stated above in the discussion of the blood urea clearance, has been found markedly less sensitive than the latter as an indicator of renal deficit. The phthalein showed one point of special interest; occasionally when an acute case, after suffering marked functional loss, began to improve, the phthalein would begin to rise some time before the blood urea clearance. We at first attempted to include the phthalein values in the charts, but under the necessity for condensation, and in view of the fact that except for the above point the phthalein shows less about renal function changes than does the clearance, we felt forced to eliminate the former.

The blood urea content, when taken without relation to the urea excretion, may, as shown by Van Slyke, McIntosh, Møller, Hannon, and Johnston (1930), like the phthalein excretion, fail to show a definite abnormality until the renal function, as measured by the blood urea clearance, has fallen to 20 per cent of normal. The same was found true of the blood creatinine content. For comparison with other data of the same sort in the literature some of the blood urea and creatinine values are given in the histories, but there was obviously no object in charting them.

Plasma fats and cholesterol have been followed in a large proportion of our cases because of the concurrence of lipemia and hypercholesterolemia with nephrosis. Plasma protein contents, however, are determinable by more accurate methods, and plasma protein deficit appears to be as closely related to the nephrotic syndrome, and more directly related to the edema, than the lipoids, hence we have not reported the latter.

The ions of the plasma, K^+, Na^+, $Cl' \cdot HCO'_3$, and H^+, show variations of great interest, especially in the terminal stage of renal failure, as shown by Austin, Peters, and their collaborators (Sunderman, Austin, and Camack, 1928; Bulger, Peters, Eisenmann, and Lee, 1926), and plasma electrolytes have been studied in our patients. However, the relationship of plasma electrolyte changes to renal failure and edema are less constant and definite than are the relationships of the urea excreting power and plasma albumin content, respectively, to these nephritic results. The interesting details of mineral metabolism have furthermore appeared incapable of treatment within the limits of the present monograph.

The basal metabolism has been determined in nearly all cases, and some of the results are given in the abstracts of case histories. The basal metabolic rates were chiefly of use in guiding treatment with thyroid substance or thyroxin in the degenerative cases, in a minority of which such treatment appeared to give beneficial results in assisting the elimination of edema.

The Urine Sediment Count of Addis

Counts of the formed elements in the sediment of the concentrated urine voided during the last 12 hours of a 24 hour dry diet have been carried out by the method of Addis (1925) on patients admitted during the past 3 years. Since, however, they are lacking in the majority of cases, these counts are not charted. In cases where they are available some of the counts are given in the case history abstracts. The normal variations in the 12-hour output of the formed elements, according to an examination of 74 students by Addis (1926) are: casts, 0 to 4200; red cells, 0 to 425,000; white blood and epithelial cells, 32,000 to 1,800,000. The normal casts are all of the hyaline variety, without any fat droplets.

Plan of Presentation of Data

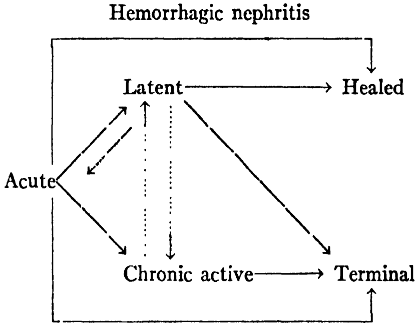
In the following pages we shall, for each of the three main types of nephropathy, give an outline of the general course of the disease, followed by a discussion of the specific changes noted as illustrated in our charts, by a discussion of the anatomical changes found at autopsy, and finally by abstracts of the histories of the charted cases, together with individual autopsy reports.

The introductory outline of the course of each disease is intended chiefly to identify, independently of the nomenclature used, the type of nephropathy concerning which specific data are presented in the subsequent section, and to indicate the nature of these data sufficiently to assist the reader in orienting himself with regard to them at the outset of their subsequent detailed discussion. The outlines are therefore brief, and in them we have made no attempt to review the literature or to present detailed clinical descriptions. These can be found in the works of Volhard and Fahr (1914) and Volhard (1918). The latter author is furthermore engaged in the preparation of a new monograph on the subject (personal communication), to which, perhaps the chemical, functional, and anatomical observations in our paper may serve as a useful supplement.

HEMORRHAGIC OR GLOMERULAR NEPHRITIS

General Course of the Disease

Addis has pictured the course of the disease by a diagram which, with slight changes,[2] is the following:



The more usual changes are indicated by arrows with unbroken shafts. The dashed arrow indicating change from chronic active to latent stage represents a favorable outcome of the chronic active which is so rare that the writers have only once observed it. Addis, however, has seen it several times (personal communication). The reversed arrow leading backwards from the latent to the acute indicates an exacerbation occurring after progress towards healing has reached the latent stage. Less frequently, an exacerbation may cause a change from the latent to the chronic active stage.

In the progress of the disease the different stages are, of course, not sharply marked off from one another. It is usually impossible to

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[2] In Addis' original paper (1925) the first stage of the disease was called "initial" instead of "acute." However, the condition produced by an exacerbation of a latent case is frequently identical in symptoms and outcome with that of a fresh, acute case. The exacerbated case resembles the fresh one in hematuria, edema, relatively rapid variability, and chance of improving to the latent condition, a chance which, according to the writers' experience, is almost nil in the chronic active stage. Since it would be inaccurate to call an exacerbated case "initial," we have retained the term "acute" to cover both initial and exacerbated cases. Otherwise we have followed Addis.

The authors have enjoyed the benefit of Addis' personal discussion and criticism of part of the following portions of this paper, and for this advantage are greatly in his debt.

EXPLANATION OF CHARTS

For the hemoglobin values two scales are given. The scale to the left gives cubic centimeters of oxygen bound by the hemoglobin in 100 cc. of blood, the "volumes per cent O_2" scale. The scale to the right gives per cent of Haldane's mean normal hemoglobin value, which is equivalent to an oxygen capacity of 18.5 volumes per cent O_2.

For the standard blood urea clearance, C_s, two scales are also given. The scale to the left shows cubic centimeter of blood cleared of urea per minute by the kidneys. The scale to the right shows the clearance values in per cent of the normal mean, which is 54 cc. per minute (Møller, McIntosh, and Van Slyke, 1928).

The plasma protein scales are obvious.

The blood pressures are given in 2 curves, the upper representing systolic, and the lower diastolic pressure, in millimeters of mercury.

For each of the above 4 values the mean normal is represented by a heavy base line. The shaded space between this line and the broken line indicating observed values shows the deviation of the latter from the average normal. When the shaded area extends downwards from the base line the observed value is below the normal average, and vice versa.

The vertical spaces which are left unshaded indicate periods during which no observations were made, usually because the patient was out of the hospital.

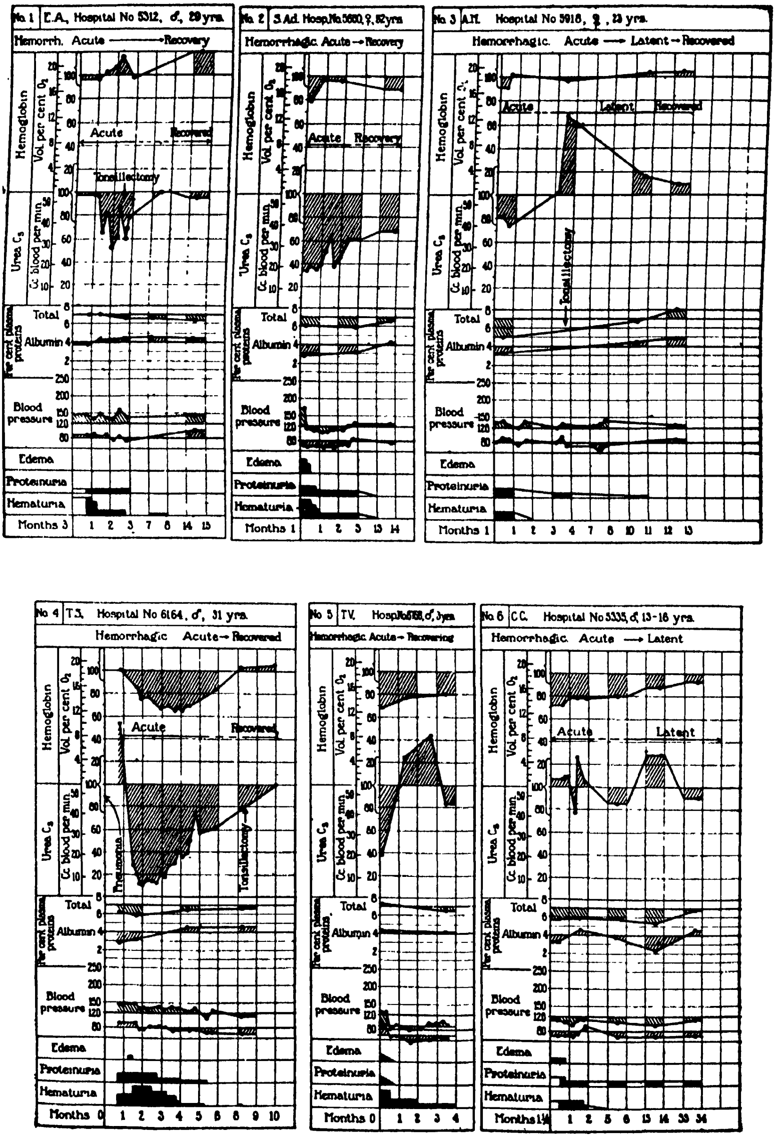
At the bottom of each chart are spaces indicating in a semi-quantitative way the amounts of edema, albuminuria, and hematuria. The black areas have the following significances.

HEIGHT OF BLACK AREA IN FIFTHS OF TOTAL SPACE	EDEMA	PROTEINURIA PER 24 HOURS	HEMATURIA
		grams	
1	Trace	Under 1	Slight microscopic
2	Moderate pitting	1 to 4	Marked microscopic
3	Marked pitting edema	Up to 10	Slight macroscopic
4	General edema with ascites	Over 10	Marked macroscopic

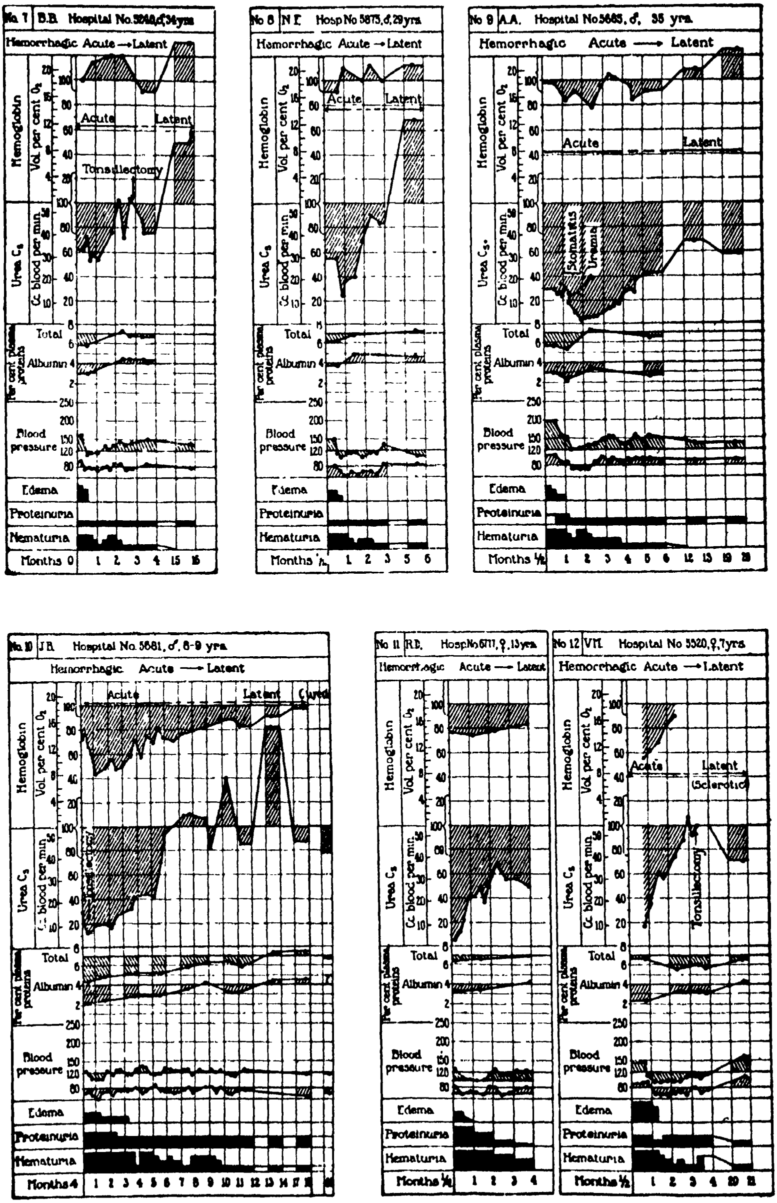
In each case for which autopsy data are presented with the case history, "Aut" is marked near the right hand margin of the chart.

At the bottom of each chart the number at the left nearest "months" indicates the number of months the disease was noted before the patient entered the hospital. The other numbers in the bottom row indicate months after first admission.

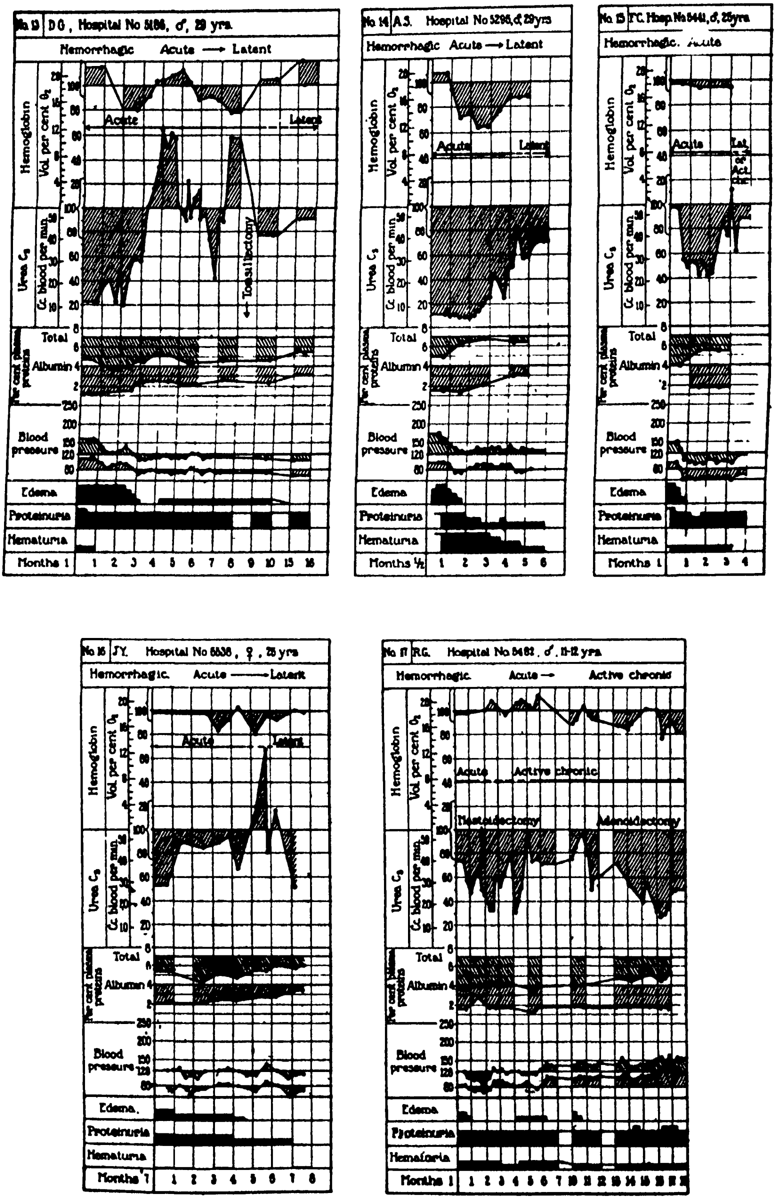
Charts of cases 1 to 50 inclusive.


No 1 E.A., Hospital No 5312, ♂, 29 yrs
Hemorrh. Acute → Recovery
Acute
Recovered
Tonsillectomy
Months 3 1 2 3 7 8 14 15
No 2 S.Ad. Hosp.No.5660, ♀, 52 yrs
Hemorrhagic Acute → Recovery
Acute
Recovery
Months 1 1 2 3 13 14
No 3 A.M. Hospital No 5918, ♀, 23 yrs.
Hemorrhagic Acute → Latent → Recovered
Acute
Latent
Recovered
Tonsillectomy
Months 1 1 2 3 4 7 8 10 11 12 13
No 4 T.S. Hospital No 6164, ♂, 31 yrs.
Hemorrhagic Acute → Recovered
Acute
Recovered
Pneumonia
Tonsillectomy
Months 0 1 2 3 4 5 6 8 9 10
No 5 T.V. Hosp.No.5768, ♂, 3 yrs
Hemorrhagic Acute → Recovering
Months 0 1 2 3 4
No 6 C.C. Hospital No 5335, ♂, 13-18 yrs.
Hemorrhagic Acute → Latent
Acute
Latent
Months 1½ 1 2 5 6 13 14 33 34
Hemoglobin
Vol per cent O₂
20 16 12 8 4
100 80 60 40 20
Urea Cs
Cc blood per min.
50 40 30 20 10
100 80 60 40 20
Per cent plasma proteins
Total
Albumin
8 6 4 2
Blood pressure
250 200 150 120 80
Edema
Proteinuria
Hematuria

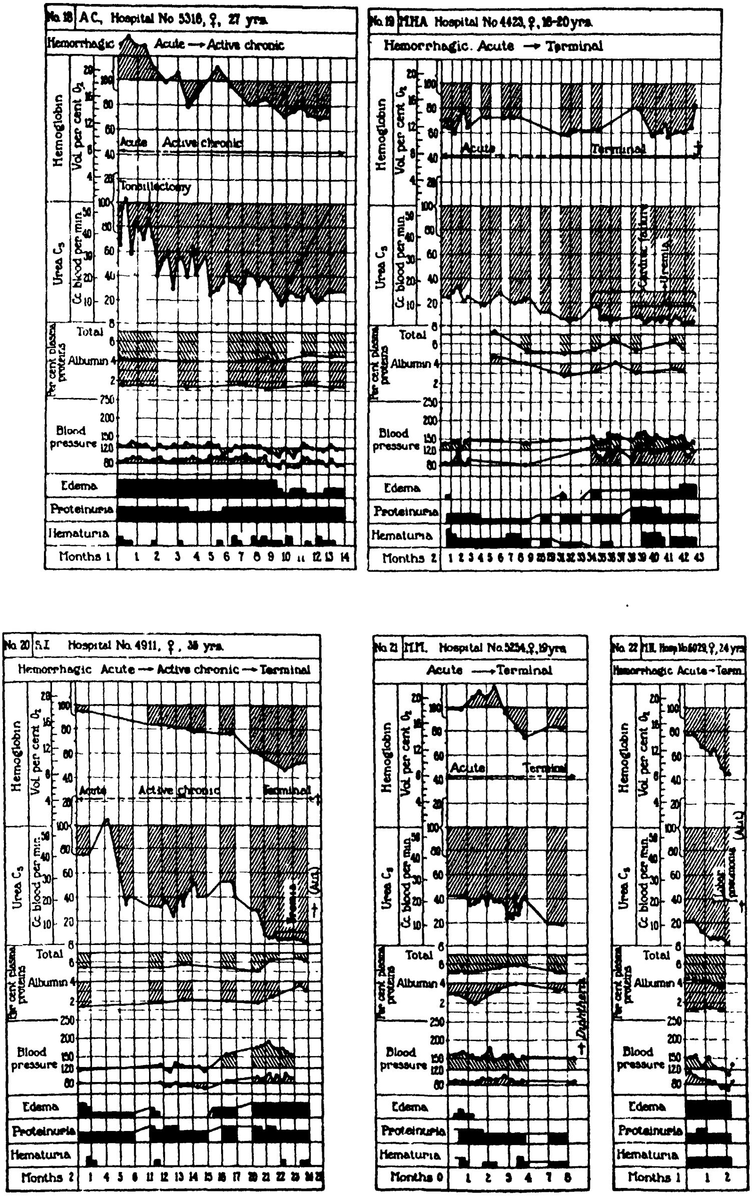
CHARTS 1–6


No 7 B.B. Hospital No. 5248, ♂, 34 yrs
Hemorrhagic Acute → Latent
Tonsillectomy
No 8 N.F. Hosp No 5875, ♂, 29 yrs
Hemorrhagic Acute → Latent
No 9 A.A. Hospital No 5685, ♂, 35 yrs
Hemorrhagic Acute → Latent
Stomatitis
Uremia
No 10 J.B. Hospital No. 5681, ♂, 8-9 yrs
Hemorrhagic Acute → Latent
Cured
No 11 R.D. Hosp No 6711, ♀, 13 yrs
Hemorrhagic Acute → Latent
No 12 V.H. Hospital No 5520, ♀, 7 yrs
Hemorrhagic Acute → Latent
(Sclerotic)
Tonsillectomy
Acute
Latent
Hemoglobin
Vol. per cent O_2
Urea C_s
Cc. blood per min.
Per cent plasma proteins
Total
Albumin
Blood pressure
Edema
Proteinuria
Hematuria
Months

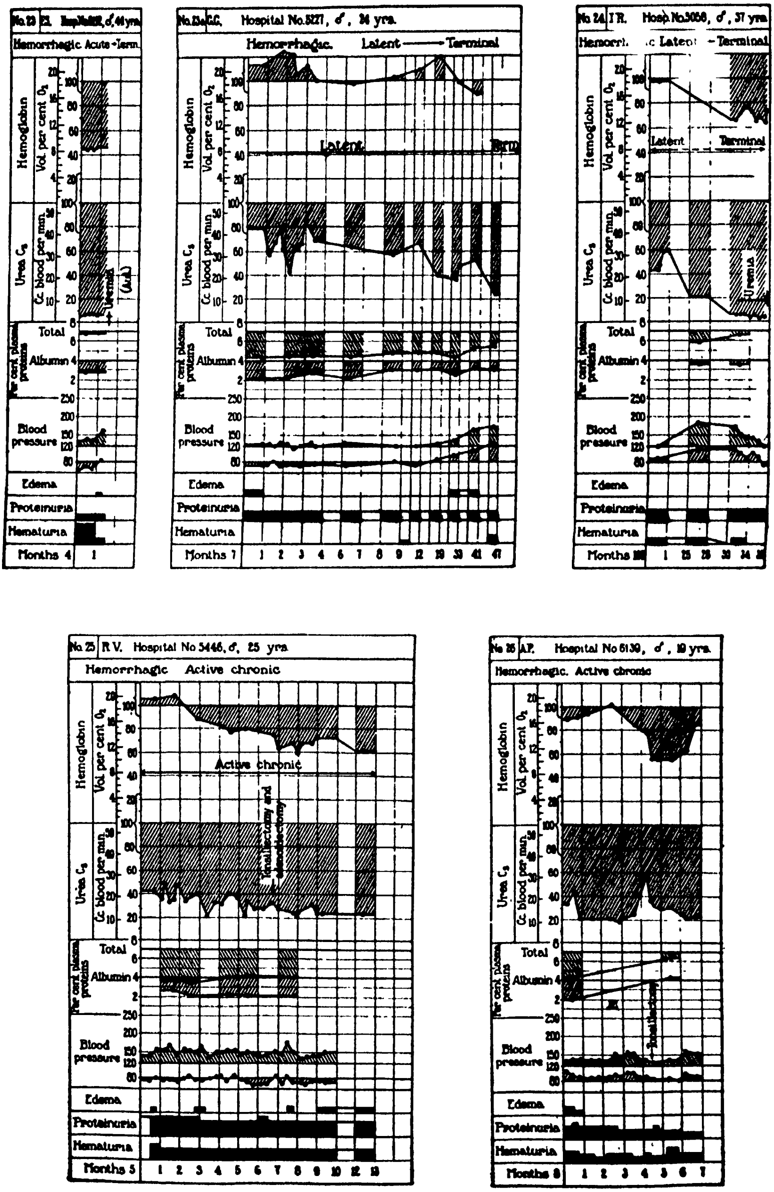
CHARTS 7–12


No 13 D G , Hospital No 5186, ♂, 29 yrs
Hemorrhagic Acute ⟶ Latent
No 14 A.S. Hospital No 5296, ♂, 29yrs
Hemorrhagic Acute ⟶ Latent
No 15 F.C. Hosp. No 5441, ♂, 25yrs
Hemorrhagic. Acute
No 16 J.Y. Hospital No 5536, ♀, 25 yrs
Hemorrhagic Acute ⟶ Latent
No 17 R.G. Hospital No 5482, ♂, 11-12 yrs
Hemorrhagic Acute ⟶ Active chronic
Hemoglobin
Vol. per cent O₂
Urea C_s
Cc. blood per min.
Per cent plasma proteins
Total
Albumin
Blood pressure
Edema
Proteinuria
Hematuria
Months
Acute
Latent
Lat. or Act. chr.
Active chronic
Tonsillectomy
Mastoidectomy
Adenoidectomy

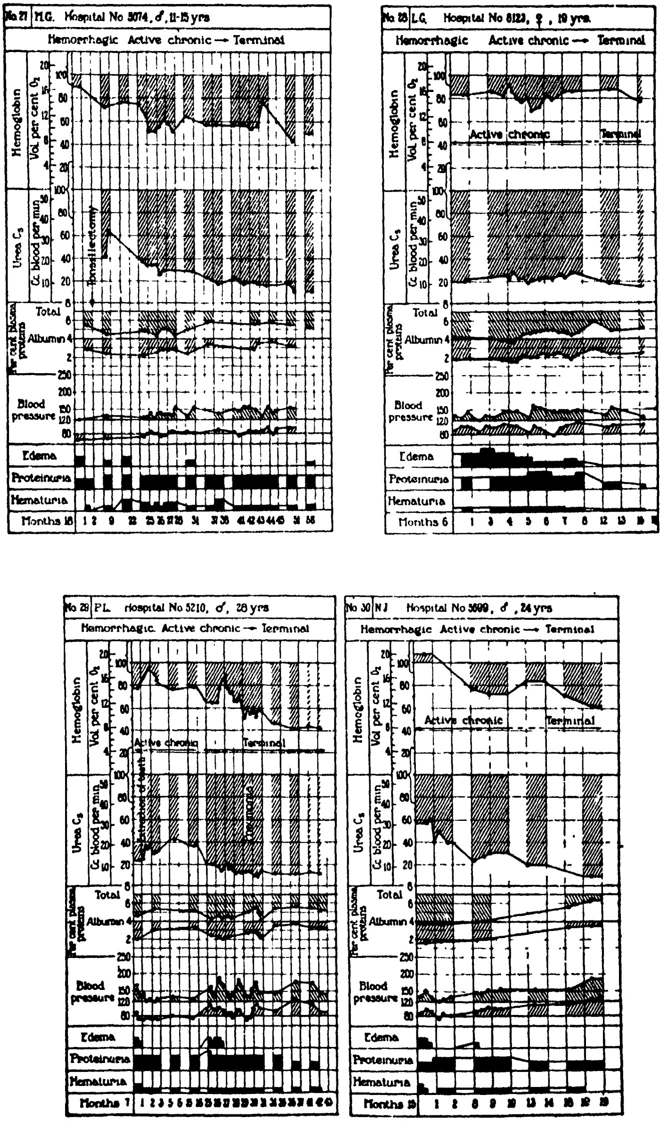
CHARTS 13–17


No. 18 A.C. Hospital No 5318, ♀, 27 yrs.
Hemorrhagic Acute → Active chronic
No. 19 M.H.A. Hospital No 4423, ♀, 18-20 yrs.
Hemorrhagic. Acute → Terminal
No. 20 S.I. Hospital No. 4911, ♀, 38 yrs.
Hemorrhagic Acute → Active chronic → Terminal
No. 21 M.M. Hospital No. 5234, ♀, 19 yrs.
Acute → Terminal
No. 22 M.H. Hosp. No. 6029, ♀, 24 yrs.
Hemorrhagic Acute → Term.
Hemoglobin
Vol. per cent O_2
Urea C_s
Cc. blood per min.
Per cent plasma proteins
Total
Albumin
Blood pressure
Edema
Proteinuria
Hematuria
Months
Acute
Active chronic
Terminal
Tonsillectomy
Cardiac failure
Uremia
Diphtheria
Lobar pneumonia
(Aut.)
†

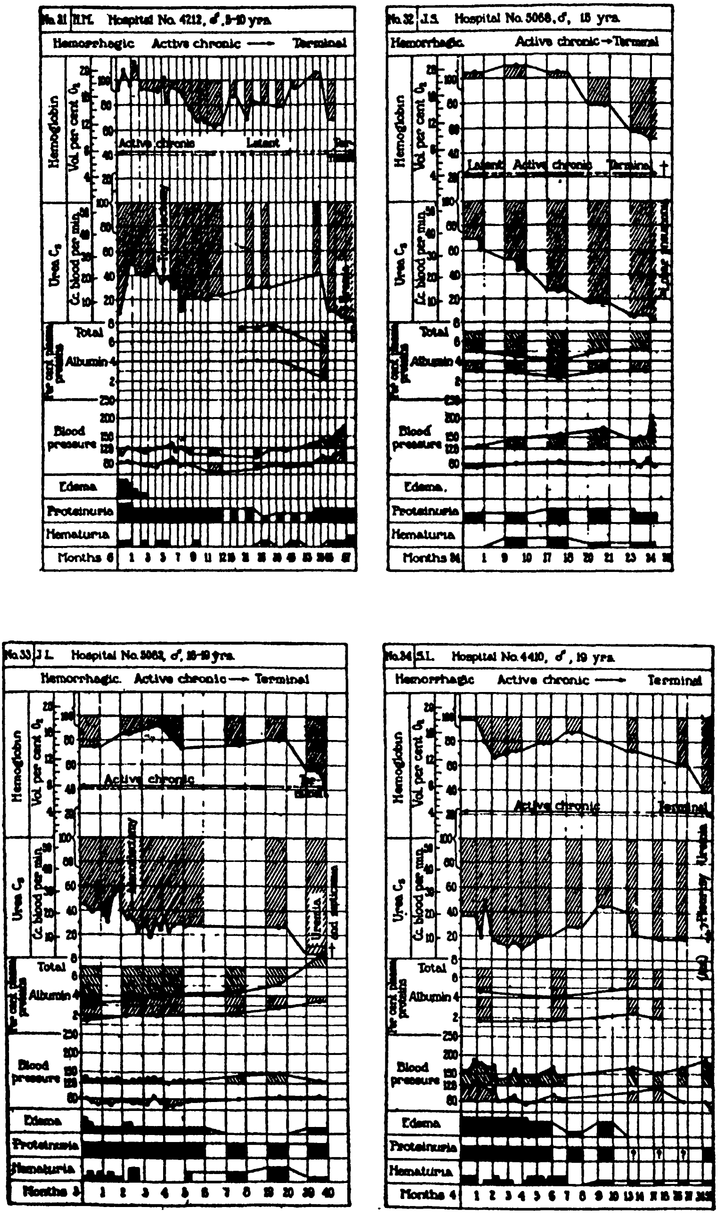
CHARTS 18–22


No. 23 E.L. Hosp. No. 5282, ♂, 44 yrs.
Hemorrhagic Acute → Term.
Uremia
(Acid.)
Months 4
No. 23a C.C. Hospital No. 5227, ♂, 34 yrs.
Hemorrhagic. Latent → Terminal
Latent
Term.
Months 7
No. 24 I.R. Hosp. No. 3058, ♂, 37 yrs.
Hemorrhagic Latent → Terminal
Latent
Terminal
Uremia
No. 25 R.V. Hospital No. 5446, ♂, 25 yrs.
Hemorrhagic Active chronic
Active chronic
Tonsillectomy and adenoidectomy
Months 5
No. 26 A.P. Hospital No. 6130, ♂, 19 yrs.
Hemorrhagic. Active chronic
Tonsillectomy
Months 8
Hemoglobin
Vol. per cent O₂
Urea C₈
Cc. blood per min.
Per cent plasma proteins
Total
Albumin
Blood pressure
Edema
Proteinuria
Hematuria

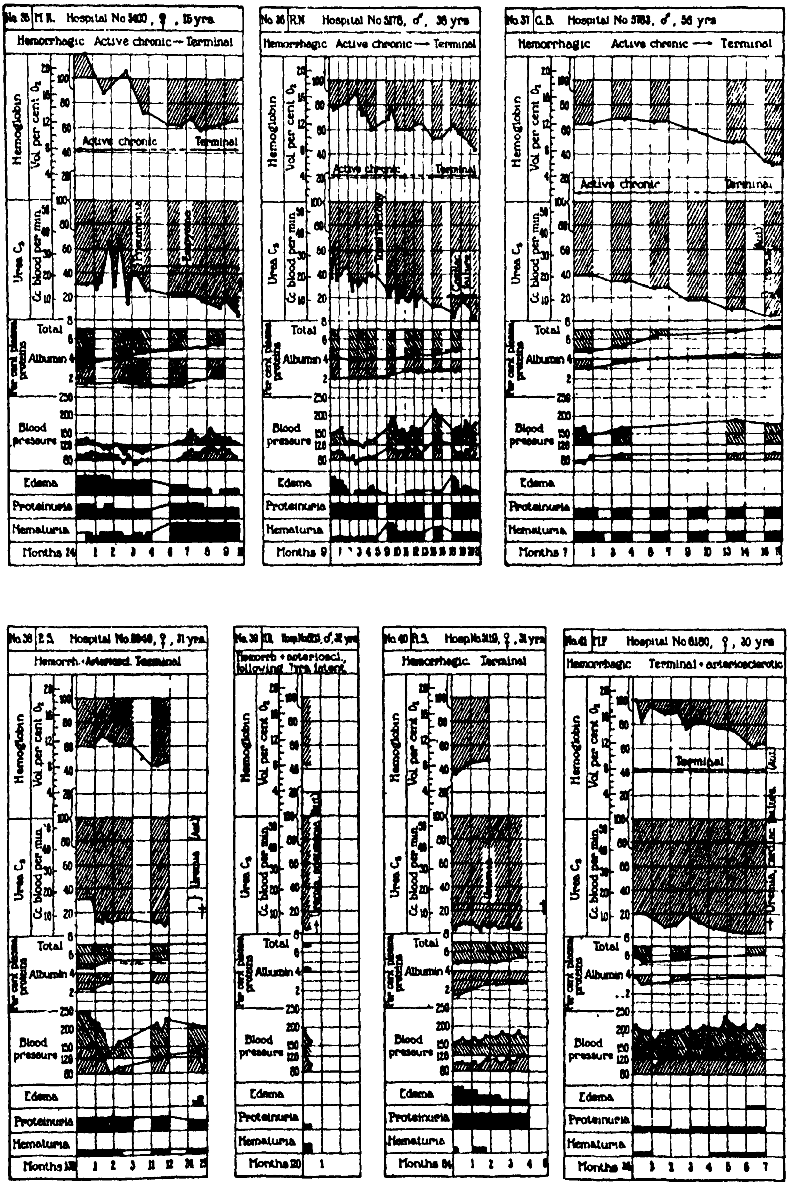
CHARTS 23–26


No 27 M.G. Hospital No 5074, ♂, 11-15 yrs
Hemorrhagic Active chronic → Terminal
No 28 L.C. Hospital No 8123, ♀, 19 yrs
Hemorrhagic Active chronic → Terminal
No 29 P.L. Hospital No 5210, ♂, 28 yrs
Hemorrhagic Active chronic → Terminal
No 30 N.J. Hospital No 5699, ♂, 24 yrs
Hemorrhagic Active chronic → Terminal
Hemoglobin
Vol per cent O2
Urea Cs
cc blood per min
Per cent plasma proteins
Total
Albumin
Blood pressure
Edema
Proteinuria
Hematuria
Months
Tonsillectomy
Active chronic
Terminal
Pneumonia

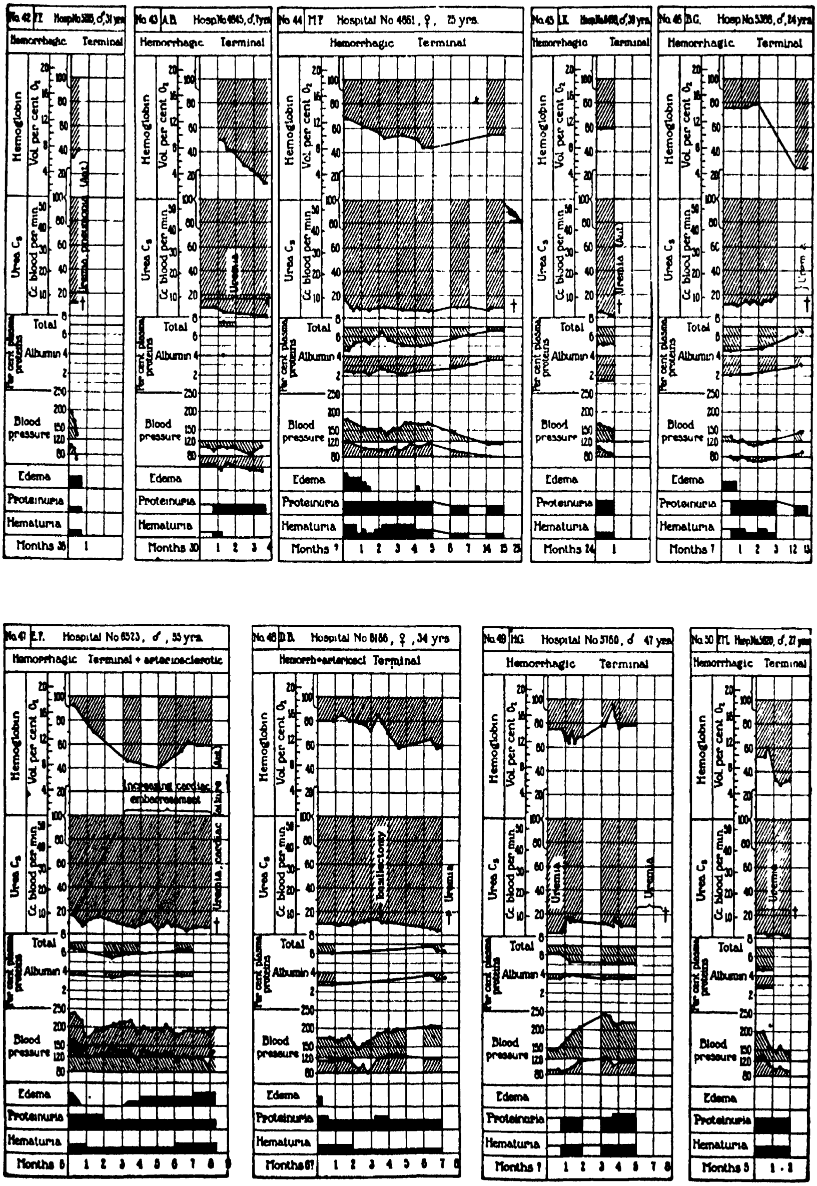
CHARTS 27–30


No. 31 H.M. Hospital No. 4212, ♂, 8-10 yrs.
Hemorrhagic Active chronic → Terminal
No. 32 J.S. Hospital No. 5068, ♂, 15 yrs.
Hemorrhagic Active chronic → Terminal
No. 33 J.L. Hospital No. 5063, ♂, 16-19 yrs.
Hemorrhagic Active chronic → Terminal
No. 34 S.L. Hospital No. 4410, ♂, 19 yrs.
Hemorrhagic Active chronic → Terminal
Hemoglobin
Vol. per cent O_2
Urea C_s
Cc. blood per min.
Per cent plasma proteins
Total
Albumin
Blood pressure
Edema
Proteinuria
Hematuria
Months
Active chronic
Latent
Terminal
Tonsillectomy
Uremia and anemia
Pleurisy
Uremia

Charts 31–34


No 35 M.K. Hospital No 3400, ♀, 15 yrs
Hemorrhagic Active chronic → Terminal
No 36 R.N. Hospital No 5176, ♂, 36 yrs
Hemorrhagic Active chronic → Terminal
No 37 G.B. Hospital No 5763, ♂, 56 yrs
Hemorrhagic Active chronic → Terminal
No 38 R.S. Hospital No 8040, ♀, 31 yrs
Hemorrh. + Arteriosc. Terminal
No 39 Hosp No 6215, ♂, 32 yrs
Hemorrh + arterioscl., following 7 yrs latent
No 40 R.S. Hosp No 3119, ♀, 31 yrs
Hemorrhagic Terminal
No 41 M.P. Hospital No 6180, ♀, 30 yrs
Hemorrhagic Terminal + arteriosclerotic
Hemoglobin
Vol per cent O₂
Active chronic
Terminal
Urea C_s
Cc. blood per min.
Pneumonia
Cardiac failure
Uremia
(Aut)
Total
Albumin
Per cent plasma proteins
Blood pressure
Edema
Proteinuria
Hematuria
Months

CHARTS 35–41


No 42 F.T. Hosp No 5185, ♂, 31 yrs
Hemorrhagic Terminal
No 43 A.B. Hosp No 4845, ♂, 7 yrs
Hemorrhagic Terminal
No 44 M.T. Hospital No 4661, ♀, 25 yrs.
Hemorrhagic Terminal
No 45 L.K. Hosp No 4680, ♂, 38 yrs
Hemorrhagic Terminal
No 46 B.G. Hosp No 5388, ♂, 24 yrs
Hemorrhagic Terminal
No 47 E.F. Hospital No 6523, ♂, 55 yrs
Hemorrhagic Terminal + arteriosclerotic
No 48 D.B. Hospital No 6166, ♀, 34 yrs
Hemorrh + arterioscl Terminal
No 49 H.G. Hospital No 5760, ♂ 47 yrs
Hemorrhagic Terminal
No 50 F.M. Hosp No 5620, ♂, 27 yrs
Hemorrhagic Terminal
Hemoglobin
Vol. per cent O₂
Urea Cₛ
Cc blood per min
Per cent plasma proteins
Total
Albumin
Blood pressure
Edema
Proteinuria
Hematuria
Months
Uremia, pneumonia (Aut.)
Uremia
Uremia (Aut.)
Increasing cardiac embarrassment
Uremia, cardiac failure (Aut.)
Tonsillectomy
†

CHARTS 42–50

state that within a certain week the acute stage changed definitely to the latent, or passed into the chronic active. Likewise the boundary between the chronic active and terminal stages is broad and hazy. The transition periods, during which the disease is passing from a condition frankly belonging to one stage to that belonging clearly to the next may cover weeks, or even months. Nevertheless the disease in its progress does pass through periods clinically so different that they have at times been defined as different entities, and the convenience of using concise characteristic names to define these stages appears to justify a differentiating nomenclature.

Acute Hemorrhagic Nephritis (Cases 1 to 23)

The acute stage with its sudden onset is familiar. It is marked regularly by hematuria, proteinuria, and edema, while diminution of renal function, hypertension, plasma protein deficit, and some anemia are frequent. Opinion seems to be coming to a general agreement that the cause is nearly always some type of streptococcus infection (Volhard and Fahr, 1914; Addis, 1925; Longcope and collaborators, 1927).

From the severity or the mildness of any of these symptoms, except plasma albumin deficit, during the first weeks of the acute stage it is, according to our data, impossible to predict whether the outcome is likely to be favorable or unfavorable.

The acute stage may have any one of four outcomes. (1) The patient may completely recover, without any detectible sign of the disease remaining (cases 1 to 5). (2) He may improve and become free from subjective symptoms of the disease, but retain some albuminuria, hematuria, or diminution in renal function, the disease becoming latent (cases 6 to 16). (3) The disease may progress into the active chronic form, almost always with subsequent more or less gradual progress to the terminal stage (cases 17, 18 and 20). (4) The disease may hasten in a few weeks directly from the acute to the terminal stage, and in a few months terminate in uremia (cases 21, 22 and 23).

The duration of the acute stage, in our cases which recovered or improved to the latent state, varied from 4 to 15 months. Noticeable improvement *began* within 4 months, in all cases that were not destined to pass into the chronic or terminal stages. In the cases which recovered most slowly (nos. 13 and 16) the last subjective symptom to disappear was the tendency to edema formation. The last clinical sign to disappear was usually the proteinuria, which frequently persisted for months after the patients were subjectively well, and after hematuria could no longer be detected by ordinary microscopic examination (cases 3, 6, 7, 9, 13, 15 and 16). Sometimes, however, microscopic hematuria has been noted as the most persistent sign.

Latent Hemorrhagic Nephritis (Cases 6 to 16, 23a, 24 and 31)

After the acute stage has lasted for a period of 2 to 4 months, unless the patient is fated to pass into the chronic or terminal condition, he begins to improve, and makes either a complete recovery, or a partial one to the latent condition, in which he is subjectively well, but in which more or less albuminuria, or slight hematuria, or a subnormal blood urea clearance, indicates that renal conditions are not quite restored to normal. Usually the blood pressure is now normal, but in an occasional case some hypertension may remain (case 12). In some cases the nephropathic signs eventually disappear, and recovery is apparently complete (case 10). Other cases may remain in the latent state for years, and eventually, sometimes as the result of an exacerbation due to infection (Addis, 1928), at other times for no tangible reason, may resume progress towards renal failure, and terminate in uremia. In cases 23a and 24 a slow fall in blood urea clearance set in after latent periods of 4 and 7 years respectively, and has already led to uremic death in case 24.

Rarely a patient reaches a latent condition as the result of arrested progress after the disease has reached the active chronic stage, sufficient renal function being maintained to permit ordinary activities. The only such case in our series is no. 31. The usual case which enters the active chronic stage passes more or less steadily downwards to the terminal.

Active Chronic Hemorrhagic Nephritis (Cases 17, 18, 20 and 25 to 37)

This corresponds in most cases to Volhard and Fahr's nephrotic type of glomerulonephritis, and to hemorrhagic chronic parenchymatous nephritis in the older American terminology.

For use in this paper we define the condition as hemorrhagic nephritis that has become chronic, with diminished urea excreting power and signs of active progress, but with blood urea clearance still above 20 per cent of normal. (For reasons stated below, we classify chronic cases with lower clearances as terminal.)

Within a period that in our cases does not exceed 4 months from the acute hemorrhagic onset, unless improvement begins the disease enters either the terminal stage, or more frequently what Addis (1925) calls the active chronic stage. The subject remains ill, malnourished, and unless under strict régime, usually edematous. Hematuria continues, but sometimes at so diminished an intensity that the red cells are detectible only microscopically by Addis' (1925, 1928) special method of examining by quantitative count the sediment of urine passed during the last 12 hours of a 24 hour dry diet. From whatever level it has emerged from the acute stage of the disease, the renal function, as measured by the blood urea clearance, maintains a progress which, observed over periods of months, usually tends steadily downwards. At best it may remain stationary for some months. In this stage improvement to the latent condition may occur, but such improvement is rare. Addis (personal communication) states that he has seen a few cases. In our series there is but one, no. 31.

The active chronic stage is usually accompanied by all the signs, described later, of degenerative renal disease, marked proteinuria, low plasma albumin and total protein content, high plasma fat and cholesterol contents, tendency to edema formation, and by the occurrence (Addis, 1925), from degenerated tubules, of epithelial cells and casts in the urine. The relationship between edema and plasma albumin deficit is that quoted above in the discussion of the significance of plasma protein content, and is the same as in nephrosis.

The differential diagnosis between this stage of hemorrhagic nephritis and the pure non-hemorrhagic degenerative Bright's disease (nephrosis) is not always easy. The active chronic stage of hemorrhagic nephritis, because of its preponderating degenerative element usually has every sign and metabolic abnormality of nephrosis. Both conditions, as Addis points out (1928) represent degenerative renal disease, the difference being that the hemorrhagic type has an additional pathological factor. This factor has been shown by Volhard and Fahr to be glomerular inflammation. The symptoms that have been used to distinguish the hemorrhagic (or glomerular) disease from pure nephrosis have been the presence of hypertension and nitrogen retention (Volhard and Fahr) and of hematuria (Addis). Hypertension, however, is absent in some cases of chronic hemorrhagic nephritis. Nitrogen retention in the chronic active hemorrhagic disease may be only relative and capable of detection when blood and urine urea contents are compared by means of the blood urea clearance determination. Also, nitrogen retention sometimes occurs in the later stages of pure degenerative renal disease, as will be shown later. Microscopic hematuria, usually constant in the hemorrhagic disease, sometimes intermittent, appears to be the most consistent single differential sign. After hematuria, hypertension appears to be the most valuable diagnostic sign. If present it may generally be accepted as ruling out nephrosis. The history often helps. History of an acute onset with hematuria is sufficient to indicate that the observed condition is a phase of the hemorrhagic or glomerular disease.

The justification for separating degenerative cases into hemorrhagic and non-hemorrhagic lies in the different prognoses. The hemorrhagic degenerative disease, once become chronic, almost regularly runs into the terminal stage described below, and uremia. The pure non-hemorrhagic degenerative disease may likewise lead to uremia, but it is more likely to continue without renal failure, and to terminate either by recovery or (more frequently in our experience) by death from intercurrent infection, either streptococcus or pneumococcus.

Chronologically the active chronic stage of hemorrhagic nephritis is intermediate between the acute and terminal stages, and usually covers a period of from 6 months to 2 years. Death usually occurs in less than 3 years after the active chronic stage is reached. We have had, however, 2 cases (nos. 21 and 31) out of 20 survive for 5 years.

Some rapidly progressing cases pass so quickly from acute to terminal that there is no intermediate stage (e.g., cases 19 and 21 of our series).

Terminal Hemorrhagic Nephritis (*Cases 19 to 24 and 27 to 50*)

With the progress of the disease the picture eventually changes from that outlined above for the active chronic stage, and assumes that of the terminal. Renal function, measured by the blood urea clearance, becomes stabilized at a lower level, and no longer shows occasional upward fluctuations. Henceforth it moves but little except for its gradual or rapid decline. The signs of degenerative disease, viz., non-cardiac edema, albuminuria, epithelial casts and cells in urinary sediment, low plasma albumin content, may continue undiminished to the uremic end, but they usually diminish and often disappear. *The picture of the uremic syndrome tends to replace that of "wet nephritis." Since the general change appears to come when the blood urea clearance has settled steadily below 20 per cent of normal, we have used that point as an arbitrary boundary between the active chronic and terminal stages.*

With the rise of plasma albumin which frequently occurs during progress into the terminal stage, the tendency to edema diminishes; when the albumin content exceeds 2.5 per cent the tendency to edema may nearly or quite disappear. At such a time the patient, because rid of the annoyance of the edema, is likely to feel subjectively improved. The renal function, even though it has fallen to 20 per cent of normal, may still be adequate to maintain freedom from toxic retention symptoms. In fact the urea and creatinine contents of the blood may remain for some time but little above the upper normal limits (Van Slyke, McIntosh, Møller, Hannon, and Johnston, 1930). This period constitutes the pre-uremic interval of the terminal stage. It may last as long as 2 years (cases 27 and 29).

When the renal function does change, however, it falls, often rapidly. Gross retention of urea, creatinine, and phosphoric acid ensues and uremia sets in, unless the latter is preceded by cardiac failure.

The duration of the disease, from the time when the blood urea clearance sank permanently below 20 per cent of normal, in the majority of our cases in which we could measure the interval, has been less than 1 year (nos. 20, 21, 22, 23, 24, 31, 32, 35, 36 and 37). In a minority it has been between one and two years (nos. 27, 29, 34, 44 and 46), and in only two has it exceeded two years (nos. 19 and 38).

CLINICAL OBSERVATIONS IN CASES OF HEMORRHAGIC NEPHRITIS (CASES 1 TO 50)

Urea Excreting Power in Hemorrhagic Nephritis

Of the 23 cases observed in the *acute period* all except nos. 3, 6, 18 and 20 showed during the first two months after onset a *fall of the* *blood urea clearance* to 50 per cent or less of normal. Remembering that the cases are ranked in order according to the gravity of their outcome, we can not conclude that maintenance of normal renal function during the first months of the acute stage justifies either a good or bad prognosis. Cases 3, 6, 18 and 20 maintained normal function during the acute stage. Of these, case 3 made an apparently complete recovery, 6 improved to the latent stage, with prospects for recovery, while 18 and 20 steadily progressed into the chronic condition, with increasing loss of function; case 20 has already died in uremia and there is no ground to hope better for 18.

Nor does it appear that we can attach prognostic significance to the *degree* of functional impairment shown during the first two months. In case 4 within two weeks of onset the clearance fell to 10 per cent of normal, yet an apparently complete recovery was made. In case 9 the clearance two and one-half months after onset fell to 4 per cent, with uremic symptoms, and was followed by recovery to at least as far as the latent condition.

The essential for a good prognosis is that within 4 months after the acute onset the clearance, if it has fallen, shall have begun a consistent climb back towards a normal level. In all of our cases that have recovered or improved to the latent condition the clearance has either remained normal or has begun to rise within this time.

The functional recovery is not always complete. In cases 9 and 11 recovery was subjectively so complete that the patients left the hospital although their clearances had reached only 50 to 60 per cent of average normal. Considering that an occasional healthy person may show clearances as low as 70 per cent of the average, however, 50 to 60 per cent indicates a satisfactory function if maintained. We have not had an opportunity to observe a latent case with such function long enough to ascertain whether the partial functional deficit was permanent, but Addis (personal communication) has seen cases in which it was so, the patients remaining stabilized, subjectively well, with only part of normal function. In such cases it appears probable that some of the glomerular damage of the acute stage has become permanent, but that a large proportion of the glomeruli have recovered, and that they may remain intact for considerable, perhaps indefinite, periods.

In all cases in which marked fall of blood urea clearance occurred during the initial months, and no definite tendency to rise followed within four months after onset, progress downwards to the active chronic or terminal stage followed (cases 18 to 23). In one case (17) such progress occurred even after a temporary return of the clearance to the normal range. In this case there was continuous active infection, necessitating two operations, one for mastoiditis and one for the removal of adenoids.

In the *chronic active period of hemorrhagic nephritis* the blood urea clearances in our cases (nos. 17 to 37) tell a single melancholy tale. The function changes are ultimately all for the worse. There may be periods of several months or even more than a year when progress is arrested. In case 31 alone was the stay sufficiently prolonged and definite to constitute an obviously latent period in the disease. This case, a boy of 6, some months after tonsillectomy was subjectively and clinically so improved that he was discharged, although his urea clearance was only 25 per cent of normal. During the next four years he lived a fairly normal life, and his clearance rose to 40 per cent normal. Then, however, it fell in some months to 10 per cent and uremia followed shortly. In all our other cases the disease has shown much shorter hesitations in its march towards terminal uremia.

In the *terminal stage*, with renal function less than 20 per cent normal there is only one outcome: this is uremia, unless exitus is hastened by cardiac failure or other intercurrent cause. The time in which the end is reached, however, varies greatly. Thus cases 22 and 23 plunged directly from acute nephritis into the terminal stage and reached uremia in 5 and 3 months after the acute onset. On the other hand case 29 lived for two years in the terminal stage and when last seen was still capable of carrying on his work as a waiter, although during the two year period his clearance gradually shrank from 20 per cent of normal to 10. He exemplifies the fact that *one can maintain bodily activity with 10 per cent of normal urea excreting power*, so long as other complications, in particular the degenerative syndrome, are not present in addition to the glomerular loss.

When, however, the blood urea clearance falls to 5 per cent of normal uremia appears to be inevitable.

Hematuria in Hemorrhagic Nephritis

Every case admitted during the first weeks of the *acute* stage showed hematuria, in most cases macroscopic. (Case 16 admitted 7 months after onset with only an occasional trace of microscopic hematuria had also a history of bloody urine at onset.) *The degree of initial hematuria appeared to have no relation to the prognosis.* Of the first four cases, which showed apparently complete recovery under observation, 3 had gross macroscopic hematuria. Nor does the period of persistence of hematuria appear to have any close relationship to the outcome. In the first three cases, those who most quickly recovered, it is true that hematuria disappeared in two to four months. A similar disappearance, however, occurred in cases 18 and 20, which went down into the active chronic and eventually the terminal stage. These latter cases showed recurrences of slight microscopic hematuria, and probably by the Addis sediment count would have shown a persistent quantitatively abnormal output of red cells. Nevertheless the hematuria assuredly subsided quickly to a slight one while the disease continued its progress at a fairly rapid rate towards a fatal conclusion.

Of the cases observed to improve to the *latent condition* (nos. 6 to 16) hematuria was observed to disappear during the periods of observation (4 to 34 months from acute onset) only in 5 of the 11. In all of the others it fell to microscopic proportions except in no. 11, who was observed for only four months, and in this case hematuria was decreasing.

Because of the fact that during improvement to the latent condition hematuria and hypertension frequently disappear before albuminuria, plasma protein deficit, and the tendency to edema show much improvement, one can easily make the error of diagnosing such a case as nephrosis, unless the history of initial hematuria is known. Cases. 13 and 16 were for several months distinguishable from pure nephrosis only by the fact that they had histories of initial hematuria.

In the *active chronic stage of hemorrhagic nephritis again the degree of hematuria appears to bear no relation to the severity or rate of progress of the disease.* Case 17 showed the heaviest hematuria of any in this stage, but the progress of his disease, as shown by his clinical condition and blood urea clearance, was not unusually rapid. Case 20 after the twentieth month of observation began to suffer a rapid decrease in clearance leading five months later to death in uremia. Yet during this period of rapid downward progress there was no hematuria detectible by ordinary microscopic examination. Case 20 was in fact for some time after admission considered to be probably a nephrosis. The occasional occurrence of microscopic hematuria, the development of a marked fall in urea clearance, and then of an increase in blood pressure, however, eventually led us to conclude that it was probably a case of hemorrhagic nephritis in which for the time the degenerative element dominated the picture, a conclusion which was confirmed by autopsy.

In the *terminal stage microscopic hematuria was present in all cases,* and determinable by ordinary examination. It was sometimes marked but usually slight, and bore no relation to the rate of progress of the clinical symptoms or the functional fall indicated by the blood urea clearance.

In our cases therefore hematuria was constant and usually gross in the acute stage. and constant but microscopic in the terminal. During the intermediate chronic active stage, and during improvement from the acute stage, hematuria may disappear, or at least become so slight that ordinary microscopic examination, without Addis' (1925) special precautions, fails to reveal it. *Cases in which hematuria thus disappears may be for a time indistinguishable from non-hemorrhagic degenerative Bright's disease (nephrosis) in every respect except their histories of acute hemorrhagic onset.*

Blood Pressure in Hemorrhagic Nephritis

Acute and latent stages. Of the 16 acute cases which recovered or improved to the latent condition (nos. 1 to 16) all except three, nos. 6, 10 and 11 showed at onset some hypertension (no. 5 showed only 130 mm. systolic but this is excessive for a three year old child). It is quite possible that nos. 6 and 10 may have had initial hypertension which disappeared by the time of admission, since the latter occurred six and four weeks after onset, respectively. No. 11, however, was admitted a week after onset with normal pressure. Of the 15 acute cases with favorable outcome on which we have early data, 13 therefore showed some elevation of blood pressure. Usually systolic and diastolic were elevated by similar heights. The systolic level reached was generally between 150 and 170 mm., but in no. 9 it approached 200.

The *duration of the hypertension* in these favorable cases was usually four to six weeks from the acute onset. In two cases, nos. 1 and 12, however, the pressure did not return permanently to normal, normal blood urea clearance being nevertheless thus far maintained. (Note: Patient No. 1 has since been examined, 2 years after the last observation noted on his chart, and his blood pressure has been found entirely normal, 118 systolic and 80 diastolic.)

Of the four cases, nos. 17 to 21, which passed during observation from acute into active chronic, three showed during the acute stage normal blood pressures, which in no. 18 persisted through the active chronic stage for at least a year, during which the renal function showed a steadily downward progress.

One can not ascribe any prognostic value to the initial blood pressure level observed during the first weeks of acute hemorrhagic nephritis. A case which shows little or no initial hypertension appears likely to pass into the chronic state as well as the one that does show hypertension in the acute stage; likewise recovery may occur in either case.

Two cases (nos. 12 and 15) showed for periods of 2 or 3 months definite hypotension, 90 to 100 mm. systolic and 40 to 60 diastolic. No. 12, a 7 year old girl, later developed a hypertension (systolic up to 200 mm.), thus far with normal urea excreting power maintained.

In the *active chronic stage* (cases 17, 18, 20 and 25 to 31), of ten cases, three showed no hypertension, while the other seven showed slight blood pressure increases, mostly in the neighborhood of 150 mm. systolic.

Of the 21 cases (19 to 50 inclusive, except 25) observed in the *terminal state of hemorrhagic nephritis* 13 showed blood pressures definitely above 150 mm. systolic, while eight did not (nos. 22, 23, 27, 28, 33, 35, 43 and 46). Most of these did show slight hypertensions, in the neighborhood of 150 systolic, but cases 33, 43 and 46 reached uremic deaths without blood pressures that could be called definitely abnormal at all.

Of the cases (nos. 38, 41 and 47) that showed systolic blood pressures over 200 mm. and came to autopsy, all showed marked arteriolar sclerosis in the kidneys.

Branch and Linder (1926) reported autopsies on 7 cases dying in uremia, with blood pressures regularly over 160/110 except during final collapse. In six of the seven arteriolar sclerosis was present.

According to the results of Branch and Linder (1926) and our own, permanent marked hypertension developing during chronic hemorrhagic nephritis usually indicates the addition of an arteriosclerotic element to the pathology of the disease. So far as can be judged by comparison of clearance changes in hemorrhagic cases with and without marked hypertension, however, the rate of renal destruction does not seem to progress significantly faster in the former than in the latter. Hypertension does indeed add its symptoms to the clinical picture, but uremia does not appear to come on much, if any, more rapidly than in cases without hypertension. Thus case 46, without hypertension or any other sign of circulatory disturbance, reached a fatal uremia in a year after his clearance was found between 10 and 20 per cent of normal; while case 38, with marked hypertension and renal arteriosclerosis (found at autopsy), lived for two years after her clearance was found at the same level.

From the above data the following conclusions appear derivable.

Hypertension occurs in most, but not all, cases with active hemorrhagic (glomerular) nephritis, acute or chronic.

In the inactive, latent cases hypertension is usually, but not always, absent.

In the terminal stage a marked rise of blood pressure occurs in many cases (e.g., nos. 24, 30, 31, 32, 36, 37, 40, 48 and 49) a few months before uremic death. Terminal cases which do not show such a change in blood pressure likewise have a bad immediate prognosis, however.

A marked *fall* in blood pressure is not uncommon in the last weeks or days of terminal nephritis in cases that have previously had hypertension (cases 24, 35, 39, 42 and 50). Such a fall is presumably a sign of heart failure.

Hemorrhagic nephritis can in exceptional cases run its entire course from acute onset to final uremia without definite hypertension. Our data on this point confirm those of Bannick (1927).

At no stage in nephritis primarily of the hemorrhagic type does blood pressure have definite prognostic significance concerning the fate of renal function or the rapidity of approach of uremia.

The definite significance that does attach to hypertension is that it marks the type of case in which death may occur from circulatory failure before renal failure has reached a lethal degree, or in which death may occur from a combination of cardiac and renal failure, as in cases 41 and 47.

Plasma Protein Content in Hemorrhagic Nephritis

In some *acute* cases the plasma proteins are practically unaffected. Such a condition appears to augur a good prognosis. Of our 23 acute cases the four which showed no fall in plasma proteins, nos. 1, 5, 8 and 11, all either recovered or improved to the latent stage.

Of the 23 acute cases, 16 maintained plasma albumin contents above 2.2 per cent. Fourteen of these 16 (nos. 1 to 14) either recovered or improved to the latent condition. Only nos. 19 and 21 failed to do so.

In seven cases the plasma albumin fell definitely below 2.2 per cent. Of these cases, 5, all except nos. 15 and 16, progressed into the active chronic stage, and nos. 15 and 16 appeared the least promising of those that improved to the latent stage.

If we draw the line for *total proteins* at 5.5 per cent the division is nearly the same, but not quite so sharp.

Unlike the urea excreting power and degree of hematuria, therefore, plasma protein content appears to be of decided prognostic significance during the acute state of hemorrhagic nephritis. *Acute hemorrhagic cases that maintain plasma albumin above 2.2 per cent, or total protein above 5.5, have a better prognosis than those that do not.* Of our cases in the group with over 2.2 per cent albumin only 13 per cent developed into progressing chronic or terminal nephritis, while of our cases in the group with less albumin 72 per cent so developed.

Cases 6 to 16 were observed *during improvement from the acute to the latent stage.* It appears that during the latent condition there is no tendency for plasma albumin deficit to develop, and that if plasma protein deficit has been retained from the acute stage slow rise of protein content to normal occurs. Such a rise is observed in cases 10, 13 and 16. From the time of acute onset until the plasma protein contents could be considered within the normal range, required 9, 17 and 20 months respectively in these three patients.

It appears probable that most cases in the *active chronic* stage develop from those which already have plasma albumin deficit during the preceding acute stage, and that the deficit developed in the acute stage is maintained in the active chronic. Examples are seen in cases 17, 18 and 20, which we were able to observe during passage from acute to active chronic.

All of the 16 active chronic cases in which plasma proteins were determined (nos. 17, 18, 20, 25 to 30 and 32 to 37) show plasma albumin deficits. In two cases (nos. 32 and 37) the albumin content fell only to about 3 per cent, but in the other 14 it was below the 2.5 per cent which appears to be the critical level for the appearance of the tendency to edema formation. The deficit in total protein, as in the acute stage, roughly parallels in most cases that in albumin, indicating that the globulin content is relatively unaffected. In case 25, however, the globulin content is also unusually low, causing a greater deficit in total protein than in albumin, while in case 20 the reverse occurs, the globulin being increased to nearly 4 per cent.

In case 31, which was admitted during the chronic active stage, there were unfortunately no plasma protein determinations done until two years later, when the case had become latent. This case was admitted during the earlier years of the work, before plasma protein determinations were routinely performed. From the massive edema and heavy proteinuria observed at admission, it appears probable that the usual plasma albumin deficit existed.

It appears evident that *a low plasma albumin content, usually below the 2.5 per cent level at which albumin deficit begins to produce edema, is the rule in the active chronic stage of hemorrhagic nephritis.*

As the active chronic stage passes into the terminal hemorrhagic there is a marked tendency for the plasma proteins to rise towards normal (cases 26, 27, 28, 29, 30, 32, 33, 35, 36 and 37). *In about half the terminal cases in our series* (see nos. 19 to 49) *the plasma proteins, both total and albumin, reached normal limits before death occurred.*

Proteinuria in Hemorrhagic Nephritis

In *acute hemorrhagic nephritis the degree of initial proteinuria appears to have no prognostic significance.* Of the 23 acute cases, the 3 which showed minimal proteinuria (nos. 1, 7 and 8) all improved; but likewise did the 3 (nos. 11, 13 and 15) who showed maximal proteinuria of more than 10 grams per 24 hours. Of the cases with intermediate degrees of protein output some recovered and some progressed.

The *duration* of the proteinuria in the cases which completely recovered (nos. 1 to 5) was from 2 weeks to 5 months.

In all the acute cases which improved to the *latent stage* (nos. 6 to 16) proteinuria persisted, but showed a marked diminution from the initial output.

In the 3 cases that passed while under observation from the acute into the *active chronic* stage (nos. 17, 18 and 20) there was no marked change in the proteinuria. In general the protein excretion in the active chronic stage is from 2 to 15 grams per day. Occasionally a single case will fluctuate over nearly this entire range within a month (data not shown on charts), but the protein content of the urine never decreases to the trace that is seen in arteriosclerotic and a few terminal hemorrhagic cases.

Terminal stage. In the 3 cases that we have seen pass directly from the acute into the terminal stage (nos. 19, 21 and 22) there has been no significant change in the protein output.

Likewise in the majority of cases passing from the chronic active into the terminal stage the protein excretion continues about the same (nos. 17, 20, 24, 25, 27, 31, 32, 33, 35, 36 and 37). However, in a considerable minority there is a decrease in protein output (nos. 26, 28, 29, 30, 39 and 44). In about one case out of five (nos. 23, 39, 41 and 42) the urinary protein sinks to a trace during the final period.

Edema in Hemorrhagic Nephritis

Acute stage. In the introductory discussion of the significance of plasma protein concentration and edema the relationship of the two has already been presented, together with the fact that an exception to this relationship occurs in the *initial period* of acute hemorrhagic nephritis, where *temporary edema occurs even in cases that do not show* *sufficient deficit of albumin in the plasma to produce of itself edema.* Cases 1 to 9 all show this type of edema, with plasma albumin above 2.5 grams per 100 cc. of plasma. In cases 5 and 8 the plasma proteins were at full normal concentration. It is evident that the initial edema of acute nephritis is attributable in part, and in some cases entirely, to some factor of this frequently toxic period other than lack of plasma albumin.

Such initial edema unaccompanied by plasma albumin deficit appears to be temporary. In all the above cases it disappeared within two months of the onset of the disease, and in case 5 within two weeks.

In cases 10, 12, 13, 14 and 16, edema disappeared at the same time that the plasma albumin showed definite increase. The increase is frequently not very marked on the charts, partly because of the small scale of the latter, partly because a relatively slight increase in the neighborhood of the critical level (albumin about 2.5 per cent) has a great effect in diminishing the tendency to store fluid.

Cases 18 and 22 exemplify persistent edema combined with continued plasma protein deficit. Salt restriction and other therapy were unsuccessful in overcoming the retention.

In cases 17 and 20 edema disappeared under treatment despite continued plasma protein deficit. It recurred at times, however, for no apparent reason, despite continual salt restriction. The tendency to edema was obviously present and fairly intense.

Latent stage. In all our latent cases (nos. 6 to 16 inclusive and 24) edema was absent, and plasma proteins were above the critical levels.

Chronic active stage. In this condition the tendency to edema is ordinarily one of the most outstanding characteristics, although a small minority of cases do not show it. Out of 17 chronic active patients in our charts 14 showed plasma albumin below 2.5 per cent. These were all either edematous, or potentially edematous and free from fluid retention only under the influence of salt restriction, sometimes reenforced by other treatment, such as daily urea administration. The correspondence between edematous *tendency* and plasma albumin deficit in this class of cases is practically uniform.

In treated cases with plasma proteins below the critical levels edema may or may not disappear. It appears to depend partly upon how low the plasma albumin sinks, partly upon other factors.

Case 25 presents an interesting example of a patient under continuous and practically uniform treatment, with plasma albumin somewhat below the critical level, who was obviously on the verge of edema, which came and went repeatedly in the course of several months. When admitted he had edema of the face, and pitting edema of the legs and sacrum; no ascites. This edema disappeared quickly under treatment. Three months after admission 3 grams of salt were added to the hitherto salt-free diet, and edema of the same sort recurred. The salt was removed, and the edema disappeared. Three months later a tonsillectomy was performed. During the month following the operation edema gradually developed, and then quickly went away. Two months later it was present again, this time without any obvious reason.

In *terminal hemorrhagic nephritis*, as shown above, plasma albumin deficit is much less common than in the active chronic stage of the disease, and edema is correspondingly less frequent. Of our thirty cases observed during the terminal stage (nos. 19 to 24 and 27 to 50) only 10 showed marked edema. Of these but 3 (nos. 22, 35 and 46) were attributable to plasma protein deficit. Four were associated with cardiac failure (nos. 19, 36, 38 and 41), and it was probably an element in 20 and 42. The remaining case, no. 33, was complicated by streptococcus septicemia at death: his plasma proteins were at this time normal. It appears that *in the terminal stage edema, when it occurs, may be due either to cardiac failure or to plasma protein deficit. In our series the number apparently due to cardiac failure was greater.*

Anemia in Hemorrhagic Nephritis

In the *acute* stage there is a considerable degree of correlation between the anemia and the degree of fall in renal function indicated by the blood urea clearance. The most marked anemias were shown by cases 4, 10, 11, 12, 14 and 19, in which the hemoglobin fell to 45 to 75 per cent of Haldane's normal average and the urea clearance fell to 5 to 25 per cent of average normal. However, blood urea clearance in case 9 fell to 4 per cent of normal and uremic symptoms became severe, without fall of hemoglobin below 80 per cent, and in case 8 the clearance fell to 30 to 40 per cent with maintenance of a perfectly normal hemoglobin content. A good hemoglobin value in acute nephritis does not therefore by any means indicate a well maintained renal function. *In prognosis the temporary anemia of the acute stage is of no more significance than the temporary fall in blood urea clearance*, which has already been discussed. And the absence of anemia is likewise no guarantee of a mild course or favorable outcome.

In the acute cases which showed anemia but later improved or recovered, the minimum hemoglobin content occurred two to four months after the acute onset.

In the *latent stage* (cases 6 to 16 and 24) anemia is absent or slight.

In the *active chronic stage* (see cases 16 to 37), as in the acute, anemia is accompanied by a decreased blood urea clearance, the value of the clearance, expressed in per cent of normal, being usually a half to a third of the hemoglobin expressed in the same way. Again, as in the acute stage, the reverse does not hold; there may be low blood urea clearance with little or no anemia. In almost every active chronic case periods are seen when the clearance was below 40 per cent normal while the hemoglobin was above 80 per cent.

With prolonged duration of low renal function the hemoglobin often shows a later and much slower fall than the clearance. Case 32 illustrates such an occurrence. The disease had apparently been latent or slowly progressing, and at the first examination the clearance was 70 per cent of normal, almost within the normal minimum, and hemoglobin was 105 per cent. During 18 months the clearance fell steadily to 28 per cent of normal, while the hemoglobin remained at 105 to 110 per cent. However, during the terminal six months following, when the clearance fell to almost zero and uremia developed, the hemoglobin fell progressively to 50 per cent.

In the *terminal stage* of hemorrhagic nephritis anemia is practically always present. When uremia is reached the oxygen capacity in our terminal cases had usually fallen to between 6 and 12 volumes per cent (32 to 64 per cent of Haldane's normal average). In case 43 the oxygen capacity fell rapidly during the final weeks and was down to 3 volumes per cent at death. On the other hand, case 49, after being for six months either on the verge of uremia or in it, died with but a relatively slight anemia of 14.5 volumes per cent oxygen capacity, 78 per cent of Haldane's normal average.

In summary, one can say that in chronic ne [illegible] e oxygen capacity of the blood seldom falls below 15 volum [illegible] without the concurrence of a marked deficit in urea excr [illegible] y, and of a condition bearing a grave prognosis. How [illegible] relationship between the degree of anemia and the loss of renal function or the probable duration of life is most irregular, occasional cases making, as shown by the progressive fall of blood urea clearance and the clinical signs, an obviously inevitable approach to uremic death with but slight fall in blood hemoglobin content. *While the presence of anemia signifies a grave prognosis, the maintenance of nearly normal hemoglobin does not gainsay the immediate onset of uremia.*

ANATOMICAL CHANGES OBSERVED IN TERMINAL HEMORRHAGIC NEPHRITIS[3]

Of the 50 cases of hemorrhagic nephritis which we report 25 died and autopsies were permitted on 11 (nos. 20, 22, 23, 34, 37, 38, 39, 41, 42, 45 and 47). Two passed into the terminal stage directly from the acute, while the other 9 first passed through more or less prolonged periods in the intermediate active chronic stage before they reached the terminal.

In every case practically all of the glomeruli were destroyed. This finding, taken with the fact that the blood urea clearance was reduced to the neighborhood of 5 per cent of normal or lower in these terminal cases, indicates the probability that *in hemorrhagic nephritis the fall in blood urea clearance is proportional to the glomerular destruction.*

Five of these cases, nos. 38, 39, 41, 45 and 47, were clinically classified, because of *marked hypertension and cardiac enlargement*, as arteriosclerotic in addition to the primary hemorrhagic disease. *All of these cases showed, in addition to the glomerular destruction, marked arteriolar changes at autopsy.*

The two cases, nos. 22 and 23, which went from the acute stage directly into the terminal, in contrast to the other nine, did not show contracted kidneys, although glomerular destruction was practically complete. The kidneys were of the large white type, and corresponded histologically with the descriptions by which Volhard and Fahr (1913) and

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[3] Detailed reports of the autopsy examinations are given below with the case histories.

Ophüls (1916) distinguished what they term the subacute or subchronic form of glomerulonephritis.[4]

Diffuse hemorrhagic glomerulonephritis is generally classified by pathologists into three stages. Volhard and Fahr (1914) and Ophüls (1916) distinguish an acute, a subchronic (or subacute) and a chronic glomerulonephritis, whereas Aschoff (1921) discriminates an acute inflammation, a stage of regeneration and reparation, and a stage of scar formation. The second stage of all writers corresponds to the large white or mottled kidneys, whereas the third stage corresponds to the secondary contracted kidneys.

It is sometimes difficult in individual cases to decide by autopsy which stage is present. This is true especially in subchronic and chronic stages. Fahr presents in his handbook (1925) such a case which anatomically resembled the second (subchronic) stage. But because of a duration of three to four years he ascribed this case to the chronic stage. Generally one might speak of a third stage (terminal chronic) when, macroscopically, the consistency of the kidneys becomes firmer and the differentiation between cortex and medulla more indistinct; when, histologically, the parenchyma becomes wasted to a large extent and the glomeruli irregular in size and lesion. In the chronic stage the kidneys may still be enlarged (as in case 20). But usually one finds all transitions of extensive shrinkage.

Except cases 41 and 47, all cases of this group were pure glomerulonephritis. *The outstanding lesions were diffuse changes of all or nearly all glomeruli.* Vascular lesions of degenerative nature were absent or insignificant.

Cases 22 and 23 showed anatomical changes characteristic for the subchronic stage. These were, as mentioned above, cases of short duration. They passed clinically directly from the acute to the terminal stage, and died with nitrogen retention three and five months respectively after acute onset. The kidneys were large and white with occasional blood dots on their surfaces. The consistency was doughy.

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[4] Our findings agree entirely with the statements of Volhard and Fahr (1914) concerning the lack of relationship between degree of functional deficit of the kidneys and the amount of gross anatomical contraction, also with their statement that the degree of contraction depends more on the duration of the disease than on the degree of functional damage that the kidneys have suffered.

The cortex was well marked off from the medulla. Histologically it was found that the renal architecture was still well preserved. There was only a slight scar formation. Case 22 belonged to the intracapillary, and case 23 to the extracapillary form. The glomeruli of case 22 were unusually rich in fibrinoid substances. Many of the tubules were degenerated in both cases. The vascular changes were insignificant. Case 23 showed only a slight cardiac hypertrophy and case 22 none at all. Both cases died with bronchopneumonia, but uremic symptoms were already present in one and, from the low level of the blood urea clearance, appeared inevitable in the other.

In the remaining cases death occurred only after the disease had run the usual more prolonged course, through the chronic active stage to the terminal. The histological changes likewise belonged to the chronic stage. Only in case 20, of two years duration, was it doubtful whether one should ascribe it, from the histology, to the second or to the third stage. The kidneys still were much enlarged and pale in color. But the consistency was already firmer, the surface was very finely granulated, and the differentiation between cortex and medulla was no longer distinct. Also, a marked cardiac hypertrophy was already present. The deciding factor for our diagnosis of a third stage (terminal at end of chronic glomerular) was the histology showing an extended destruction of the renal architecture (fig. 1). The outstanding lesion of the glomeruli was a diffuse hyalinization of the loops.

Case 42 is a further example of beginning shrinkage. On the one hand, the kidneys were already decreased in weight and their surfaces were slightly granulated. But, on the other hand, the kidneys were still of doughy consistency and their cortex was well marked off from the medulla. That the disease here was in the chronic stage was shown by the histology, which greatly resembled that of case 20. Also, a marked cardiac hypertrophy was found.

Cases 20 and 42 died in uremia.

The remaining 5 pure cases (nos. 34, 37, 38, 39 and 45) showed advanced contracted kidneys, which, in most instances, were grayish in color and showed both fine and coarse granulations. All were firm in consistency. The differentiation between cortex and medulla was indistinct or missing. The cases all showed marked cardiac hypertrophy and all died in uremia, except one who died with confluent bronchopneumonia. Histologically, these older cases differed from those which passed directly from acute to terminal stage by the extensive destruction of the renal architecture and the greater irregularity of the glomerular changes which had occurred during the longer disease. Besides numerous entirely hyalinized glomeruli there were glomeruli which still contained one or more loops filled with blood.

As might be expected from the variety of the lesions, certain features were found which bestowed an individual aspect upon each case. In cases 34 and 38, the glomeruli were still very large and rich in nuclei, whereas in case 37 the hyalinization of the glomerular loops was outstanding. In cases 39 and 45 there was a striking slight proliferation and desquamation of glomerular epithelial cells which, according to Fahr (1925), gives the impression of a more rudimentary inflammation.

In the wasted parenchyma, islands of better preserved tubules were seen, as is well shown by figure 11. The dilatation of these tubules is looked upon as an attempt at compensation, whereby the organism seeks to enlarge the secreting surface. Some of the epithelial cells of these tubules showed fatty degeneration, whereas hyaline-droplet degeneration was rare or missing.

The vascular changes varied a great deal in these cases. An advanced intimal hyperplasia was seen in cases 38, 39 and 45. An endarteritis was found in all cases, but reached higher degrees only in cases 38 and 39, which during life showed exceptionally high blood pressures. In case 38 also some necroses in the walls of the arterioles were seen. Hyalinization and fatty degeneration were present in all cases. But they were only slight in most instances and were not comparable to the changes which are usual in arteriolosclerosis.

Among the eleven cases autopsied in the hemorrhagic group nos. 41 and 47 were not pure cases of glomerulonephritis. The glomeruli of both cases were diffusely changed, mainly by hyalinization of the loops. But in addition both cases showed an extensive hyalinization and fatty degeneration of the arterioles, and advanced intimal hyperplasia of the medium sized and larger arteries and an endarteritis with some necroses of the small arteries. Case 41 furthermore showed a fresh pyelonephritis.

That in hemorrhagic nephritis we have to deal with a glomerulonephritis is shown by the presence regularly of diffuse disease of the glomeruli. (It is improbable that in case 41 the fresh pyelonephritis was responsible for the diffuse hyalinization of the glomeruli. According to all we know the glomerular lesions were much older than the pyelonephritis.) However, it is more difficult to decide whether the arterio- and arteriolosclerosis in cases 41 and 47 was of secondary nature or whether it existed independently of the glomerulonephritis. We are inclined to believe that it did so exist and that we have to deal in cases 41 and 47 with combined primary and secondary contracted kidneys. Aside from the fact that the arterio-, and, especially, the arteriolosclerosis, were of a high degree, the macroscopical granulation, partly caused by islands of compensatory dilated tubules, was also partly caused by scarred retractions of the parenchyma. This point is emphasized by Aschoff (1921). Furthermore, we found an advanced atherosclerosis also in other parts of the bodies of these two cases.

ABSTRACTS OF HISTORIES AND AUTOPSY REPORTS OF CASES OF HEMORRHAGIC BRIGHT'S DISEASE

A brief abstract is given of each case history covering some significant points not shown in the charts.

For each case in which an autopsy was obtained a report is also given of the post mortem observations, with microphotographs of kidney sections. Autopsies were obtained in cases 20, 22, 23, 34, 37, 38, 39, 41, 42, 45 and 47.

Case 1. Hospital No. 5312. E. A., male, 29 years. *Hemorrhagic Bright's disease.* Acute → recovery.

Three months before admission he had tonsillitis and otitis, followed by edema. Albuminuria and a blood pressure of 140 mm. were found.

On admission the otitis had cleared up. Some tonsillitis was found. The urine sediment contained many erythrocytes and erythrocyte casts and a few granular casts. The heart size was normal. Wassermann reaction was negative. Blood urea nitrogen 21 mgm. per cent, blood creatinine 1.64 mgm. per cent and the basal metabolic rate −6.6 per cent. During observation the casts disappeared. The tonsils were removed without exacerbation of the Bright's disease.

Patient was re-examined 4 years after first admission. Blood pressure was 116/80 and all urinary findings negative. Nephritis apparently completely healed.

Case 2. Hospital No. 5650. S. A., female, 52 years. *Hemorrhagic Bright's disease.* Acute → recovery.

One month before admission she had a severe cold followed by empyema in the left antrum. One week before admission hematuria was found.

On admission the urine sediment contained many erythrocytes and leucocytes, some granular and erythrocyte casts. The eyegrounds were normal. Heart somewhat enlarged. Culture of the washings from the antrum showed hemolytic streptococci. Wassermann reaction was negative. Blood urea nitrogen 22 mgm. per cent, blood creatinine 1.59 mgm. per cent.

The sinus cleared up under local treatment. She was in good condition when discharged. When seen 13 months after admission the urine sediment and blood pressure were both normal.

Case 3. Hospital No. 5918. A. M., female, 23 years. *Hemorrhagic Bright's disease.* Acute → recovery.

One month before admission peritonsillar abscesses on both sides were followed by edema. Albumin, blood, and casts were found in the urine.

On admission chronic tonsillitis was found. The urine sediment contained many erythrocytes and erythrocyte casts, some leucocytes and hyaline, epithelial, and granular casts. The eyegrounds were normal. The heart was not enlarged.

During observation the number of cells and casts in the urine decreased rapidly. Seven months after admission the casts had disappeared completely.

Three months after admission the tonsils were removed, without exacerbation of patient's nephritis. She returned to hospital for examination a year after her first admission. There was no albuminuria, but a concentrated 12-hour specimen contained 1,482,000 red cells, 946,000 white and epithelial cells, and 210,000 hyaline casts. The blood pressure was 100/68.

Case 4. Hospital No. 6164. R. S., male, 31 years. *Hemorrhagic Bright's disease.* Acute → recovery.

The patient was admitted because of a severe pneumonia, caused by pneumococcus Type II (atypical). During the convalescence from pneumonia an acute nephritis developed, and in the urine sediment many erythrocytes and erythrocyte casts appeared. Some hyaline and granular casts were present also during the pneumonia. Only few leucocytes were present and no doubly refractive bodies. The eyegrounds were normal. The heart size was normal. Wassermann reaction was negative. Blood urea N, 12 mgm. per cent, and plasma cholesterol, 250 mgm. per cent. During the pneumonia the blood creatinine was found to be 1.45 mgm. per cent.

In the next 2 weeks the blood urea nitrogen increased to 97 mgm. per cent and the blood creatinine to 6.29 mgm. per cent. Two weeks later the blood creatinine was 3.75 mgm. per cent. The sediment still showed some erythrocytes and erythrocyte casts. The tonsils were removed 6 months after the onset of disease, without causing a recrudescence. One month later a concentrated 12-hour specimen of urine contained no albumin, and 5,500,000 red cells, 782,000 white blood and epithelial cells, and 54,000 hyaline casts, in a 12-hour collection. The disease was considered to be latent.

Case 5. Hospital No. 6768. T. V., male, 3 years. *Hemorrhagic Bright's disease.* Acute → recovery.

The patient had had chronic bronchitis for one and one-half years. Two weeks before coming to hospital he had developed bilateral otitis media. Two days before admission gross hematuria had occurred which was accompanied by oliguria.

On admission the patient showed slight pitting edema of the lower extremities. Gross hematuria was present. Blood casts were seen in the sediment. The protein excretion in the urine was 5 grams during the first 24 hours. The blood urea nitrogen was 19 mgm. per cent. The plasma cholesterol was 319 mgm. per cent. A phenolsulphonephthalein test revealed 72 per cent return of the dye in 2 hours. The total protein of the blood plasma was 6.9 per cent.

Two days after admission the patient suffered an attack of hypertensive crisis—resulting in a temporary right hemiplegia from which he recovered rapidly. Gross hematuria disappeared on the seventeenth day. The protein excretion in the urine dropped to a trace during the first month. Blood pressure levels both systolic and diastolic dropped to normal during the first week. During the first two weeks a loss of weight (edema) to the extent of 4 kgm. occurred.

During the subsequent 4 months that the patient was observed, the number of red cells in concentrated 12-hour urine specimens became rapidly less and on discharge but 2,000,000 were found in urine passed during the last 12 hours of a 24-hour dry diet (normal is 500,000). The number of casts and white blood cells were reduced in the same proportions. Tonsillar tabs and adenoids were removed as added precaution against continued activity of the kidney lesion. The plasma proteins remained normal. The blood urea nitrogen varied between 8.1 and 19 mgm. per cent. When discharged the presence of a quantitatively slight hematuria was the only evidence of renal disease. Examination 3 months later, 8 months after onset, showed normal blood pressure, renal function and urine sediment, or apparently a complete recovery as far as could be demonstrated.

Case 6. Hospital No. 5335. C. C., male, 13 to 16 years. *Hemorrhagic Bright's disease.* Acute → latent.

Six years before admission he had acute nephritis with much edema. The attack cleared up in two months. Since then he has felt in perfect health. The urine has not been examined. Six weeks before admission hematuria and slight edema appeared.

On admission the urine sediment contained many erythrocytes and a few hyaline and granular casts. The heart size was normal. Blood urea nitrogen 22 mgm. per cent and blood creatinine 1.29 mgm. per cent.

During observation the number of casts in the sediment decreased and disappeared at the same time as the erythrocytes. The blood pressure, blood urea nitrogen and creatinine remained normal.

Four months after discharge there were still a few red cells, and hyaline casts in the urine, along with a trace of albumin. Two years after discharge, the urine showed only a very faint trace of albumin, the sediment contained very occasional red cells. The blood pressure remained normal.

Case 7. Hospital No. 5240. B. B., male, 34 years. *Hemorrhagic Bright's disease.* Acute → latent.

The last 5 years before admission he had recurrent tonsillitis. Three weeks before admission he had a peritonsillar abscess. Two days before admission edema appeared.

On admission chronic tonsillitis was found. The urine sediment contained many erythrocytes and some hyaline and granular casts. The heart was not enlarged. Wassermann reaction was negative. Blood urea nitrogen 50 mgm. per cent, blood creatinine 1.57 mgm. per cent, and the basal metabolic rate −7.1 per cent.

The number of casts decreased gradually and they disappeared between 3 and 15 months after admission. The blood urea nitrogen rapidly fell to normal. A tonsillectomy was performed, and was followed by a heavy hematuria, which cleared up in a few days. Several re-examinations showed only traces of albumin in the urine. There was still a faint trace 15 months after his first admission, although the sediment was then negative, and the blood pressure normal.

Case 8. Hospital No. 5875. N. F., male, 29 years. *Hemorrhagic Bright's disease.* Acute → latent.

For 3 weeks before admission the patient felt weak and chilly. Three days before admission edema appeared and albumin and casts were found in the urine.

On admission the urine sediment contained many erythrocytes, a few leucocytes and hyaline, granular and erythrocyte casts. The heart size was normal. Wassermann reaction was negative. Blood urea nitrogen 37 mgm. per cent and blood creatinine 1.87 mgm. per cent.

In a few days the blood pressure came down to normal. After the first 2 months of observation the urine sediment showed only few erythrocytes and single hyaline and granular casts. When he was seen 5 months after onset of disease, there were still red cells, and occasional red cell and granular casts to be found. Albuminuria amounted to only a very faint trace.

Case 9. Hospital No. 5665. A. A., male, 35 years. *Hemorrhagic Bright's disease.* Acute → latent.

For many years several of his teeth have been infected and alveolar abscesses have formed. Two weeks before admission he had lumbar pain and edema; albuminuria and hematuria appeared.

On admission the urine sediment contained many erythrocytes, some leucocytes and hyaline and granular casts. The eyegrounds showed blurring of the outlines of the discs and fresh retinal hemorrhages. The heart was somewhat enlarged to the left. The gums showed gingivitis, from which Vincent's organism was obtained. Wassermann reaction was negative. Blood urea nitrogen 68 mgm. per cent, blood creatinine 1.73 mgm. per cent.

Three weeks after admission the gingivitis grew worse. For about a week there was nausea, headache and a distinct drowsiness. The blood urea nitrogen rose to 209 mgm. per cent and the creatinine to 10.84 mgm. per cent. Under local treatment the mouth cleared up, the general condition improved and in 3 months the blood urea nitrogen and creatinine decreased to normal values. Twelve and 20 months after admission the patient was in good condition and able to work 6 hours daily. The casts had decreased gradually in number and had disappeared 20 months after admission. There was albumin in the urine, amounting to 0.18 gram per liter.

Case 10. Hospital No. 5681. J. B., male, 8 to 9 years. *Hemorrhagic Bright's disease.* Initial → latent → recovered.

He has had frequent attacks of tonsillitis since the age of 4. Four months before admission edema appeared. Three months later gross hematuria was noticed.

On admission chronic tonsillitis was found. The urine sediment contained many erythrocytes, leucocytes, and granular and erythrocyte casts. The heart size was normal. Wassermann reaction was negative. Blood urea nitrogen 79 mgm. per cent and blood creatinine 1.90 mgm. per cent.

Tonsillectomy was performed 3 weeks after admission. Improvement in the Bright's disease followed almost at once (see chart). The number of erythrocytes in the sediment decreased, the erythrocyte casts disappeared and the granular casts were much reduced in number. Since 5 months after admission the blood urea nitrogen and creatinine have shown normal values. Two years after admission, improvement was still taking place. There was 0.25 gram of albumin per liter in the urine, and in the sediment a few red cells, and a very few hyaline casts. The case had entered the latent phase. Three and one-half years after admission signs of renal diseases were entirely negative.

Case 11. Hospital No. 6777. R. D. F., female, 11 years. *Hemorrhagic Bright's disease.* Acute → latent.

The patient had a history of sore throats for several years—tonsillectomy and adenoidectomy had been performed 4 years previously. One year before admission a sore throat had occurred accompanied by cervical adenitis. One week previous to admission adenitis again occurred. Five days later and two days before admission gross hematuria appeared.

On admission to hospital hematuria and generalized edema were present. The blood urea N was 114 mgm. per cent. Plasma NPN 128 mgm. per cent. A phenolsulphonephthalein test revealed but a 12 per cent return of the dye in two hours. The protein excretion in the urine was 8 grams daily.

During the first month that the patient was under observation the blood urea N fell to 21 mgm. per cent. A drop in protein excretion to 1 gram per day and a decrease in weight of 6 kgm. also occurred, after which no edema was observed. Gross hematuria continued. The phenolsulphonephthalein excretion rose to 34 per cent in two hours.

During the subsequent three months the blood urea N varied between 10 and 20 mgm. per cent. Gross hematuria disappeared during the second month although red blood cells and blood casts were present in concentrated urine specimens in large numbers. Proteinuria decreased to 0.2 gram daily during the fourth month. The 2-hour phthalein excretion rose to 67 per cent before discharge from the hospital four months after onset.

Case 12. Hospital No. 5520. V. M., female, 7 years. Height 113.7 cm. *Hemorrhagic Bright's disease.* Acute → latent with hypertension.

Two weeks before admission edema appeared and albuminuria was found.

On admission chronic tonsillitis was found. The urine sediment contained many erythrocytes and erythrocyte casts, a few leucocytes and some hyaline and granular casts. Blood urea nitrogen 145 mgm. per cent and blood creatinine 2.61 mgm. per cent.

During the first month of observation the blood urea nitrogen and blood creatinine decreased to normal values. The urine sediment did not change significantly except for a decrease in the number of erythrocytes and erythrocyte casts. Tonsillectomy was carried out, and did not appear to influence the course of the Bright's disease. When the 7-year old patient was seen 20 months after admission blood pressure had risen to 160/110 and generalized arteriosclerosis and cardiac hypertrophy were noted. There were still occasional red cells in the urine, but no note of casts; albumin was only a very faint trace. The Bright's disease was considered to be latent, and the cardio-vascular disease occupied the foreground.

Case 13. Hospital No. 5186. D. G., male (colored), 29 years. *Hemorrhagic Bright's disease.* Acute → latent.

One month before admission edema was noticed.

On admission chronic tonsillitis was found. The urine sediment contained a few erythrocytes, some leucocytes and many hyaline and granular casts. The eyegrounds were normal. The heart was somewhat enlarged. Wassermann reaction was negative. Blood urea nitrogen 38 mgm. per cent, blood creatinine 1.86 mgm. per cent and plasma cholesterol 231 mgm. per cent. Five months after admission the basal metabolic rate was −16.7 per cent. The blood pressure soon fell to normal. The number of casts gradually decreased somewhat. The blood urea nitrogen remained normal; 4 months and 7 months after admission it showed minimum values of 12 and 16 mgm. per cent respectively. Patient was discharged after 10 months, subjectively well. Six months later he was still well, despite continued proteinuria, and his plasma protein content had improved.

Case 14. Hospital No. 5295. A. S., male, 29 years. *Hemorrhagic Bright's disease.* Acute → latent.

Two weeks before admission the patient had peritonsillar abscesses followed by edema.

On admission chronic tonsillitis was found. The urine sediment contained many erythrocytes and leucocytes, some hyaline and granular casts and a few erythrocyte casts. The eyegrounds were normal. The heart was somewhat enlarged. Blood urea nitrogen 149 mgm. per cent. Blood creatinine, 2.0 mgm. per cent on admission, rose during 3 weeks to 4.9 mgm.

During observation the number of erythrocytes, leucocytes and casts in the urine decreased very much, but a few of each were still present 5 months after admission. The blood urea nitrogen and creatinine became normal about 3 months after admission. Patient returned to work as chauffeur, and refused to come back to hospital for subsequent examinations.

Case 15. Hospital No. 5441, F. C., male (colored), 25 years. *Hemorrhagic Bright's disease.* Acute → latent.

Five years before admission he had primary syphilis. He never had any secondary or tertiary symptoms. For 5 weeks before admission he had had bronchitis and for four and a half weeks edema.

On admission the urine sediment contained some erythrocytes, a few leucocytes, and many granular casts. The heart was not enlarged. Wassermann reaction was strongly positive. Blood urea nitrogen 21 mgm. per cent and blood creatinine 1.30 mgm. per cent. The basal metabolic rate was −6.1 per cent.

During observation the erythrocytes disappeared from the urine sediment, and the number of casts decreased. Blood pressure, blood urea nitrogen, and creatinine remained normal. The last 4 weeks of observation the patient had daily inunctions of mercury. He reported himself in good health two and one-half years after admission; consequently it is probable that his nephritis had either healed or become latent. He could not return for examination.

Case 16. Hospital No. 6538. J. Y., female, 25 years. *Hemorrhagic Bright's disease.* Acute → latent.

No history of previous infections. Eight months before admission hematuria had appeared, followed by edema. Hematuria had disappeared in two months, and edema had become more pronounced.

On admission pitting edema of the lower extremities was present. A 12-hour concentrated specimen of urine showed 1,900,000 red blood cells, 3,000,000 white blood and epithelial cells, and 125,000 hyaline casts. The protein excretion in the urine was 3.5 grams daily. The blood urea N was 17 mgm. per cent. The phenolsulfonephthalein test revealed a 54 per cent return of the dye in two hours.

During the 8 months that the patient was under observation the blood pressure remained normal. The phthalein excretion rose to above 60 per cent in 2 hours and remained above that level. In concentrated 12-hour specimens the urine sediment showed a variation in red blood cells between 150,000 and 2,860,000, the white blood cells and epithelial cells between 1,500,000 and 4,000,000, and the hyaline casts between 60,000 and 250,000. The protein excretion diminished to 0.5 gram daily at the end of the observation period. At no time was the blood urea nitrogen above 17 mgm. per cent.

Case 17. Hospital No. 5482. R. G., male, 11 to 12 years. *Hemorrhagic Bright's disease.* Acute → active chronic.

Two years before admission he had mastoiditis. He was operated on and a draining sinus remained. Three weeks before admission edema appeared and albumin and casts were found in the urine.

On admission the open pus-secreting mastoid process lesion was seen. The urine sediment contained many erythrocytes, some leucocytes and hyaline and granular casts and a few erythrocyte casts and doubly refractive bodies. The eyegrounds were normal. The heart was not enlarged. Blood urea nitrogen 29 mgm. per cent and blood creatinine 1.24 mgm. per cent. During the second month in hospital a secondary mastoid operation was done, and satisfactory healing occurred.

During observation there was no significant change in the urine sediment, plasma proteins, blood urea nitrogen or creatinine. The case was evidently an active chronic one when discharged 18 months after first observation.

Case 18. Hospital No. 5316. A. C., female, 27 years. *Hemorrhagic Bright's disease.* Acute → active chronic.

Five weeks before admission edema appeared and albuminuria was found.

On admission chronic tonsillitis was found. The urine sediment contained many erythrocytes, a few leucocytes and some hyaline and granular casts. The heart size was normal. Wassermann reaction was negative. Blood urea nitrogen 20 mgm. per cent, blood creatinine 1.36 mgm. per cent and the basal metabolic rate −9.5 per cent.

Some time after admission a bleeding urethral polyp was found. It was removed by operation 5 months after admission. Erythrocyte casts were recorded twice. The urine sediment did not change significantly; occasionally doubly refractive bodies were found. The blood pressure remained normal. The blood urea nitrogen and creatinine increased slowly; 13 months after admission they were 36 and 2.08 mgm. per cent respectively.

Case 19. Hospital No. 4423. M. A., female, 16 to 20 years. *Hemorrhagic Bright's disease.* Acute → terminal → exitus.

Two months before admission albuminuria was accidentally found, and shortly afterwards edema appeared.

On admission an abscess of the right gum and several badly decayed teeth were found. The urine sediment contained many erythrocytes, few leucocytes, and many granular and a few erythrocyte casts. The eyegrounds were normal. Wassermann reaction was negative. Blood urea nitrogen was 28 mgm. per cent and plasma cholesterol 255 mgm. per cent.

During the 3½ years observation the blood urea nitrogen and creatinine gradually rose. After 2 years they were 89 and 3.2 mgm.; after 3 years 253 and 6.0 mgm. and 12 days before death they were 328 and 8.1 mgm. per 100 cc. respectively.

Death occurred in uremia and cardiac failure. The autopsy showed cardiac hypertrophy and contracted kidneys with extensive chronic hemorrhagic Bright's disease.

Case 20. Hospital No. 4911 and 5386. S. J., female (colored), 35 years. *Hemorrhagic Bright's disease.* Acute → active chronic → terminal → death.

Two months before admission edema appeared.

On admission the urine sediment contained some leucocytes, occasional erythrocytes and many hyaline and granular casts. The eyegrounds were normal. The heart was not enlarged. Blood urea nitrogen was 9 mgm. per cent. Thirteen months after admission the basal metabolic rate was −6.0 per cent.

During observation the blood urea nitrogen increased gradually and was 132 mgm. per cent two weeks before death, which occurred in uremia.

Autopsy. No. 249/1926. Autopsy commenced 9 hours after death.

Death in uremia. Cardiac hypertrophy. Small fresh areas of bronchopneumonia. Old adherent pleurisy and pericarditis. Old perisplenitis and perihepatitis. Ascites. Chronic cholelithiasis. Slight arteriosclerosis of the spleen. Slight atheromatosis of the aorta.

The right kidney weighed 165, the left 220 grams. The capsule stripped with difficulty. Both kidneys were firm. Their surface was very finely granulated and pale in color. Hemorrhages were absent. The cortex was of about normal width and pale in color. The differentiation of cortex and medulla was not sharp. The right kidney contained at the upper pole a cyst the size of an acorn.

Microscopic examination. All glomeruli are more or less diseased (fig. 1). Their loops are changed mostly into thick hyaline or protoplasmatic masses the lumina of which frequently can no longer be recognized (fig. 2). Part of the glomeruli show increased numbers of nuclei and are poor in erythrocytes. Occasionally one finds small necroses of the loops (fig. 2). Hemorrhages in the capsular spaces were not seen. Nearly all glomeruli are adherent to the capsules, often to a large extent. The capsules are mostly rich in nuclei and thickened by masses stained red by Van Gieson. Frequently one sees crescents rich in nuclei. The parietal epithelial cells are often increased in number and size. Fairly many glomeruli are entirely hyalinized.

The parenchyma is much destroyed (fig. 1) and penetrated by scars which are rich in collagenous connective tissue and in capillaries dilated with blood. Frequently they contain round cell infiltrations and occasionally polymorphonuclear leucocytes, which may enter the tubules. Xanthoma like cells were not seen. The tubules of these regions are in all stages of atrophy and degeneration up to entire disappearance. Between the scars one sees islands of better preserved tubules which are much dilated. They contain coagulated protein, some desquamated epithelial cells, some casts and in some places many polymorphonuclear leucocytes (fig. 1). Occasionally one sees calcified casts. The epithelial cells are mostly flattened. In single tubules one sees also hyaline-droplet degeneration. Occasionally one finds distinct signs of regeneration.

Some arterioli show an endarteritis. The larger arteries show a well developed intimal hyperplasia. The medulla is rich in collagenous connective tissue. In some places it contains scars rich in capillaries dilated with blood. The tubules are frequently atrophic or degenerated and contain an exudate similar to that of the cortex.

Anatomical diagnosis. Chronic glomerulonephritis. Secondary arteriosclerosis.

Case 21. Hospital No. 5254. M. M., female, 19 years. *Hemorrhagic Bright's disease.* Acute → death from diphtheria.

For some years she has had frequent colds. One week before admission edema appeared.

On admission the urine sediment contained some erythrocytes, leucocytes and granular casts and a few hyaline casts. The eyegrounds were normal. The heart was not enlarged. Wassermann reaction was negative. Blood urea nitrogen 26 mgm. per cent. Blood creatinine 1.58 mgm. per cent.

During observation the number of casts increased somewhat. The blood urea nitrogen during the first 4 months was about 35 mgm. per cent and was 80 mgm. per cent 11 months after admission. The blood creatinine at this time was 3.64 mgm. per cent. Eleven months after admission the patient died at home from diphtheria. No autopsy.

Case 22. Hospital No. 6029. M. K., female, 24 years. *Hemorrhagic Bright's disease.* Acute → uremic death.

Five weeks before admission she had diarrhoea and vomiting. One week later edema was called to her attention.

On admission considerable gingivitis was found. The urine sediment contained some erythrocytes, leucocytes, doubly refractive bodies, and hyaline and granular casts. The heart was somewhat enlarged. Wassermann reaction was negative. Blood urea nitrogen 58 mgm. per cent. Blood creatinine 1.76 mgm. per cent.

During observation vomiting and nausea were present. The number of casts increased, and some of these were broad (Addis' renal failure casts). The blood urea nitrogen rose to 170 mgm. per cent and blood creatinine to 2.67 mgm. per cent. The patient then contracted atypical pneumonia, caused by Type IV pneumococcus. One day before death the blood urea nitrogen was 418 mgm. per cent and blood creatinine 4.54 mgm. per cent. The autopsy showed pneumonia, pancreatic fat necrosis, cardiac hypertrophy, large mottled kidneys, with extensive hyaline degeneration of the glomeruli and in some places crescent formation. There was tubular atrophy and degeneration.

Autopsy. Autopsy No. 281/1927. Autopsy commenced 3 hours after death. Death by confluent bronchopneumonia. Empyema of the right pleural cavity. Moderate cardiac hypertrophy. Serous pericarditis and ascites. Fibrinous perisplenitis. Old healed endocarditis mitralis. Moderate atheromatosis of the aorta, aortic cusp of the mitral valve and of the coronary arteries.

The right kidney weighed about 200, the left 210 grams. The capsule stripped easily. Both kidneys were doughy in consistency. In each kidney the surface was relatively smooth with very fine, slight granulations. It was yellowish white in color and contained occasional red dots. The cortex was a little enlarged and in color similar to the surface. Fine radiate brownish stripes were visible in it. The cortex was fairly well marked off from the brown red medulla.

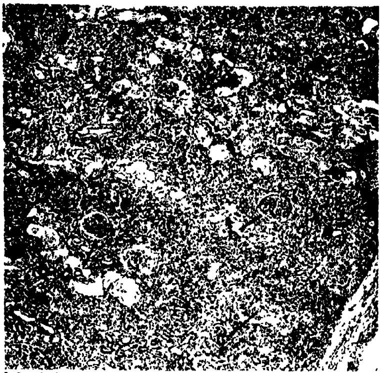
Microscopic examination. All glomeruli are more or less severely changed (fig. 3). Their tufts are partly or entirely changed into thick hyaline or fibrinoid masses which are in part stained blue by Gram (fig. 4). The nuclei of such tufts are often necrotic and bizarrely shaped. Other parts of these glomeruli contain *greatly increased numbers of nuclei* (fig. 3) partly of proliferated endothelial cells and partly of polymorphonuclear leucocytes. Occasionally one sees karyokinetic figures. The tufts are thrombosed in some instances. Part of the glomeruli are enlarged. Their tufts extend frequently into the tubules like hernias. They are extremely poor in erythrocytes. In some instances one sees adhesions of the tufts to the capsules. In the glomerular spaces one sees a little coagulated protein and detritus. The capsules are well preserved in most instances. Occasionally the parietal epithelial cells are increased in number. A few capsules are thickened by masses stained red by Van Gieson. Relatively frequently one sees also crescents which occasionally are very thick and consist of several layers, rich in nuclei. These changes are, however, insignificant in contrast to the intracapillary changes. Several glomeruli are entirely hyalinized. The more severely changed glomeruli contain in part much fat which occasionally is doubly refractive.

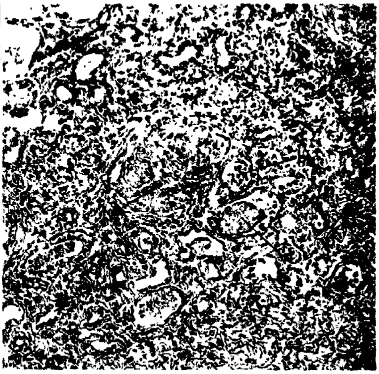
The parenchyma is penetrated by a few scars which are rich in collagenous connective tissue and capillaries dilated with blood. Frequently they contain round cell infiltrations. The tubules of these regions are in all stages of atrophy and degeneration up to entire disappearance. The better preserved tubules are frequently dilated and contain varying amounts of coagulated protein, polymorphonuclear leucocytes and desquamated epithelial cells. Occasionally they contain hyaline casts. The epithelial cell of the ascending loops are swollen in part and frequently fatty degenerated. Hyaline-droplet degeneration is rare and insignificant. In some places one sees signs of regeneration.

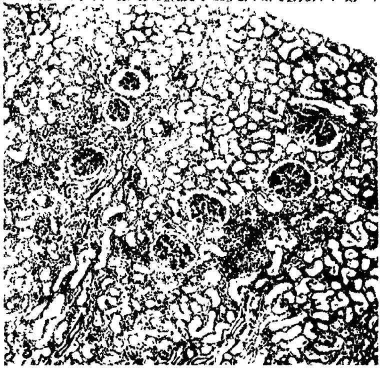
The arterioli are mostly well preserved. Occasionally they show a moderate endarteritis. The larger arteries show a moderate intimal hyperplasia.

Anatomical diagnosis. Subacute glomerulonephritis. Intracapillary form of Fahr.

Case 23. Hospital No. 6112. F. S., male, 44 years. *Hemorrhagic Bright's disease.* Acute → terminal → exitus.







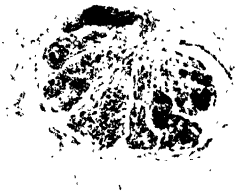


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FIG. 1. Case 20. Anatomical diagnosis: chronic glomerulonephritis. Clinical diagnosis: terminal hemorrhagic nephritis.

The tufts of the glomeruli are clotted with hyaline and protoplasmic masses and are frequently adherent to the capsules. Some of the capsules are much thickened (*a*). The covering cells of the tufts are increased in number (*b*). The parenchyma is greatly destroyed and penetrated by scars which contain round cell infiltrations. The remaining tubules contain coagulated protein, polymorphonuclear leucocytes, (*c*), and occasionally calcified casts. Zenkers, iron-hematoxylin-eosin. ×60.

FIG. 2. Case 20. For diagnosis see figure 1.

The tuft of the glomerulus is changed into thick protoplasmic and hyaline masses the lumina of which can no longer be recognized. In (*a*) one sees a small necrosis. The tuft is adherent to the capsule in many places. The capsule is thickened. In the surrounding tubules and interstitial tissue one sees some polymorphonuclear leucocytes (*b*). In (*c*) desquamated epithelial cells and cellular detritus in a tubule. Zenkers, iron-hematoxylin. ×200.

FIG. 3. Case 22. Anatomical diagnosis: subacute glomerulonephritis. Clinical diagnosis: terminal hemorrhagic nephritis developed directly from acute.

The glomeruli are enlarged and rich in endothelial cells and polymorphonuclear leucocytes. Part of their loops are necrotic and fibrinoid or hyaline. In a few places one sees adhesions of the tufts to the capsules. The tubules contain coagulated protein and some polymorphonuclear leucocytes. In the interstitial tissue a few scars with infiltrations of round cells and a few polymorphonuclear leucocytes. Mueller-formalin fixation, iron-hematoxylin-eosin. ×60.

FIG. 4. Case 22. For diagnosis see figure 3.

The loops of the glomerulus are clotted with fibrinoid masses and are adherent to the capsule. Mueller-formalin fixation, Gram. ×200.

The patient 2 years before admission had been told that his urine contained no albumin and that his blood pressure was normal. Four months prior to coming to hospital, he had contracted a severe cold which had been followed by arthritis. There had been no gross hematuria or edema.

On admission the patient was in an uremic condition. There was severe anemia. The hemoglobin was 8.8 volume per cent O_2 capacity. There was no edema. The heart was not enlarged. The urine contained a quantity of albumin, red blood cells, white blood cells, and casts. Many of the casts were of the broad renal failure type (Addis, 1925). The blood urea N was 117 mgm. per cent, the creatinine 9.9 mgm. per cent. The phthalein output was but 2 per cent in 2 hours.

During the 2 months that the patient was under observation, the blood urea N remained between 91 and 117 mgm. per cent. The creatinine stayed at 9 mgm. per cent. The phthalein excretion was reduced during the second month to a trace in two hours. The hemoglobin was constant at 8 volumes per cent O_2 capacity. The excretion of albumin, red blood cells and casts continued. Slight edema appeared a few days before death. There was a slight elevation of the systolic blood pressure level during the last month, the diastolic level remained normal. The patient died in uremic coma.

Autopsy. Autopsy No. 283/1927. Autopsy commenced 3 hours after death. Death by bronchopneumonia. Fresh serous fibrinous pleurisy. Old adherent pleurisy of the left cavity. Serous pericarditis Ascites. Old adherent perisplenitis and perihepatitis. Old healed endocarditis. Old infarcts in the spleen. Moderate atheromatosis of the aortic cusp of the mitral valve, of the coronary arteries and of the aorta.

Neither the right kidney nor its ureter and arteries could be found. The left kidney was very large. Length 16, breadth 8 cm. The capsule stripped with difficulty. The kidney was doughy in consistency. Its surface was smooth and occasionally slightly granulated. It was yellowish white in color showing red blood dots. The cortex was much enlarged showing a picture similar to that of the surface. The cortex was well marked off from the medulla.

Microscopic examination. All glomeruli are severely diseased (fig. 5). Their tufts are entirely clotted by hyaline masses (fig. 6) stained red by Van Gieson in most instances. Nearly all glomeruli are empty of blood and show partially increased numbers of nuclei. Occasionally one sees necroses of the loops. Nearly all glomeruli are adherent to the capsules to a large extent (fig. 5). In the capsular spaces one finds some coagulated protein and erythrocytes in places. The covering cells of the glomeruli as well as the parietal epithelial cells are much increased in number and show *frequently typical crescents* (fig. 6). The capsules are thickened and cannot be sharply differentiated from the surrounding tissue.

The tubules are frequently dilated and contain some coagulated protein, casts, some erythrocytes and polymorphonuclear leucocytes. Their epithelial cells are much desquamated. Some are flattened. Others are swollen and occasionally show hyaline-droplet degeneration. The interstitial tissue is thickened in some instances and contains round cellular infiltrations (fig. 6). The arterioli are relatively well preserved. The larger arteries show a slight intimal hyperplasia.

Histological diagnosis. Subacute glomerulonephritis. Extracapillary form of Fahr. Aplasia of the right kidney.

Case 23a. Hospital No. 5227. G. G., male, 24 years. *Hemorrhagic Bright's disease.* Latent → terminal.

The patient had a heavy cold a year before admission. He first noted edema about the ankles 5 months later, and albumin was disovered in the urine. Examination disclosed nothing abnormal beyond a low grade of chronic tonsillitis, and slight edema. The heart was normal in size. The urine contained about 4 grams of albumin per day. There were many hyaline casts, and some granular casts in the sediment. A few red and a few white blood cells were occasionally noted, and fatty epithelial cells. Blood urea N was 12 mgm. per cent. Blood creatinine 1.64 mgm. per cent. Plasma cholesterol 217 mgm. per cent.

The edema cleared up quickly, and did not recur until 2 years later. There has been a definite downward progress in renal function during the last 2 years, and marked hypertension, 175/125, and hematuria have developed. The case is apparently passing from the latent stage, where it resembled a mild nephrosis, into the terminal stage of hemorrhagic nephritis.

Case 24. Hospital No. 5058. I. R., male, 37 years. *Hemorrhagic Bright's disease.* Latent → terminal → exitus.

Nine years before admission he had acute nephritis. One year later he had a relapse, and this time gross hematuria was noticed. Since then albuminuria has persisted. He never noticed any edema.

On admission pyorrhea alveolaris and chronic bronchitis were found. The urine sediment contained a few erythrocytes and some hyaline and granular casts. The eyegrounds were normal. The heart size was normal. Blood urea nitrogen was 53 mgm. per cent, blood creatinine 2.0 mgm. per cent and the basal metabolic rate +1.8 per cent.

The pyorrhea improved after treatment. Twenty-five months after admission headaches, nausea and hypertension were present. Blood urea nitrogen was 102 mgm. per cent and blood creatinine 2.75 mgm. per cent. Thirty-four months after admission retinal hemorrhages were present, and the heart was enlarged. The blood urea nitrogen was 291 mgm. per cent and blood creatinine 14.4 mgm. per cent. The maximal values reached were 910 mgm. per cent and 20.32 mgm. per cent respectively. Patient died in uremia. No autopsy.

Case 25. Hospital No. 5446. R. V., male, 25 years. *Hemorrhagic Bright's disease.* Active chronic → terminal.

He has had rather frequent colds. Five months before admission after a severe cold edema and dyspnoea appeared. One month later tonsillectomy was performed.

On admission there was no dyspnoea. Large adenoids and remnants of tonsillar tissue with foci of pus were present. The urine sediment contained many erythrocytes, leucocytes, and hyaline and granular casts, also some cellular casts and doubly refractive bodies. The eyegrounds were normal. The heart was somewhat enlarged. Wassermann reaction was negative. Blood urea nitrogen 39 mgm. per cent. Blood creatinine 2.13 mgm. per cent. Six months after admission the basal metabolic rate was +6.6 per cent. Nine months after admission, the adenoids and tonsillar tags were removed. No improvement occurred after the operation, nor did the urine sediment and blood pressure at the time change significantly. Twelve months after admission the blood urea nitrogen was 69 mgm. per cent and the blood creatinine 3.70 mgm. per cent. The patient was then up and about and working 6 hours each day, despite the progressive decrease of renal function.

Case 26. Hospital No. 6139. A. P., male, 19 years. *Hemorrhagic Bright's disease.* Active chronic.

The patient had chronic tonsillitis for many years. Eight months before admission edema appeared, and one month later albuminuria, hematuria and hypertension were found. All these signs were present when he was admitted to this hospital.

On admission the urine sediment contained many erythrocytes, some leucocytes and hyaline casts, many erythrocyte casts and occasional doubly refractive bodies. The eyegrounds were normal. Chronic tonsillitis was found. The heart size was normal. Wassermann reaction was negative. Blood urea nitrogen 35 mgm. per cent. Blood creatinine 1.86 mgm. per cent. The tonsils and adenoids were removed, without any subsequent flare up in the Bright's disease. A month later, he was allowed up, and an increase in hematuria occurred, lasting about a week.

Case 27. Hospital No. 5074. M. G., male, 11 to 13 years. Height 138.5 to 145.5 cm. *Hemorrhagic Bright's disease.* Active chronic → terminal.

He had pneumonia at 6, 7 and 10 years of age. After the last attack, 18 months before admission, edema and ascites appeared and albuminuria was found.

On admission chronic tonsillitis and chronic bronchitis were found. The urine sediment contained occasional erythrocytes and granular casts. The heart was somewhat enlarged. Wassermann reaction was negative. Blood urea nitrogen 18 mgm. per cent. Blood creatinine 1.48 mgm. per cent. Basal metabolic rate +3.6 per cent.

Fourteen months after admission the urine sediment contained many erythrocytes and granular and cellular casts and a few leucocytes, and these findings have persisted. On several occasions some doubly refractive bodies have also been found. The blood pressure most of the time has been about 140/80. Intermittent chronic bronchitis has persisted. Twenty-four months after admission chronic frontal and ethmoidal sinusitis appeared, caused by hemolytic streptococcus. The blood urea nitrogen has been about 60 mgm. per cent most of the time and the blood creatinine about 1.80 mgm. per cent. The plasma proteins have not changed significantly. He was still able to be active over 6 years after the onset of his disease, and a year and a half since it has been in the terminal stage. In that time, however, his blood urea clearance has sunk from 20 to 12 per cent of normal.

Case 28. Hospital No. 6123. L. G., female, 19 years. *Hemorrhagic Bright's disease.* Active chronic → terminal.

Six months before admission edema appeared. One month later marked albuminuria and hematuria were found. At the Lenox Hill hospital the basal metabolic rate was found to be −32 per cent and the plasma cholesterol 375 mgm. per cent.

On admission here the urine sediment contained some erythrocytes, leucocytes and hyaline and granular casts. The eyegrounds were normal. The heart size was normal. Wassermann reaction was negative. Blood urea nitrogen 71 mgm. per cent. Creatinine 1.33 mgm. per cent. Plasma proteins were low and edema general, with ascites. Four months after admission the plasma cholesterol was found to be 1113 mgm. per cent. During observation no significant change was found in the urine sediment.

Case 29. Hospital No. 5610. P. L., male, 28 years. *Hemorrhagic Bright's disease.* Active chronic → terminal.

Seven months before admission moderate edema gradually appeared.

On admission abscesses were found at the roots of two teeth. The urine sediment contained some erythrocytes and leucocytes and hyaline, epithelial and granular casts. The eyegrounds were normal. The heart size was normal. Wassermann reaction was negative. Blood urea nitrogen 44 mgm. per cent. Blood creatinine 1.62 mgm. per cent.

Plasma albumin increased gradually until it approached normal levels. The urine sediment did not change much except for the appearance of broad casts 25 months after admission. Sixteen months after admission a gradual rise began in the blood urea nitrogen and creatinine. Thirty-four months after admission they were 213 mgm. per cent and 8.58 per cent respectively, blood urea clearance 12 per cent of normal. Lobar pneumonia occurred 29 months after admission caused by pneumococcus Type III. He made a good recovery. When seen 41 months after his first admission, he had no complaints, his blood urea clearance was still 12 per cent of normal, and his general appearance was very good. He had then been 7 months at home unrestricted with regard to activity and diet.

Case 30. Hospital No. 5699. N. J., male, 23 years. *Hemorrhagic Bright's disease.* Active chronic → terminal.

The last 3 years before admission he had several attacks of tonsillitis. After such an attack 15 months before admission edema appeared and albuminuria was found. The condition improved under treatment, but the patient developed psoriasis, for which he was treated with chrysarobin. Two days later the edema reappeared and was present until admission. Three months before admission tonsillectomy was performed. After this the systolic blood pressure rose gradually from normal to 145 mm.

On admission some patches of psoriasis were present. The urine sediment contained some erythrocytes, a few leucocytes and hyaline casts, and many granular casts. The eyegrounds were normal, the blood pressure about 135/90, the heart somewhat enlarged to the left. Wassermann reaction was negative. Blood urea nitrogen 23 mgm. per cent. Blood creatinine 1.93 mgm. per cent.

The edema and psoriasis disappeared rapidly. Since 2 months after admission the patient has been able to do clerical work. Twenty months after admission he had begun to suffer from attacks of headache and vomiting, and the blood pressure had then increased to 188/126.

Case 31. Hospital No. 4212. H. M., male, 5 to 10 years. Height 105 to 120 cm. *Hemorrhagic Bright's disease.* Active chronic → terminal → exitus.

One brother of the mother died of acute uremia and another had acute hemorrhagic nephritis.

Six months before admission edema appeared and albuminuria was found. Both have persisted.

On admission chronic tonsillitis was found. The urine sediment contained few erythrocytes and many granular casts. The eyegrounds were normal. The heart was not enlarged. Wassermann reaction was negative. Blood urea nitrogen 52 mgm. per cent. Plasma cholesterol 780 mgm. per cent.

Between the 54th and 60th months after admission blood creatinine rose from 2.1 to 3.7 mgm. per cent and blood urea N from 67 to 234 mgm. per cent. Two days before death in uremia the figures had risen to 17.0 and 403 mgm. respectively No autopsy.

Case 32. Hospital No. 5068. J. S., male, 15 years. Height 176 cm., ideal weight 61.3 kgm. F = 0.99. *Hemorrhagic Bright's disease.* Active chronic → terminal → exitus.

Two years before admission he had scarlet fever followed by acute nephritis with hematuria, hypertension and edema. Since then persistent albuminuria and intermittent edema have been present.

On admission the urine sediment contains very few hyaline and granular casts. The heart size was normal. Blood urea nitrogen 20 mgm. per cent. Basal metabolic rate +3.2 per cent.

During observation the number of casts increased. At the terminal stage broad casts are present. The systolic blood pressure rose steadily and reached 202/76 before death. The diastolic pressure was never found to be increased. Enlargement of the heart was first noticed 17 months after admission. The blood urea nitrogen and creatinine increased gradually and were 341 and 10.7 mgm. per cent respectively, 4 days before death. Plasma albumin oscillated between 2.32 and 3.08 per cent. No definite signs of uremia were present before death. No autopsy.

Case 33. Hospital No. 5082. J. L., male, 16 to 20 years. *Hemorrhagic Bright's disease.* Active chronic → terminal → exitus.

Three months before admission edema appeared and albuminuria was found. On admission adenoid hypertrophy was found. The urine sediment contained some erythrocytes and leucocytes and hyaline, granular, and cellular casts. The eyegrounds were normal. The heart size normal. Wassermann reaction was negative. Blood urea nitrogen 47 mgm. per cent. Creatinine 1.51 mgm. per cent. Plasma cholesterol was 457 mgm. per cent two months after admission. Thirty-nine months after admission the patient returned to hospital, complaining of weakness, vomiting and itching. He died 8 days later, from uremia, and a septicemia due to streptococcus hemolyticus. No autopsy.

Case 34. Hospital No. 4410 and 5055. S. L., male, 18 to 20 years. *Hemorrhagic Bright's disease.* Active chronic → terminal → exitus.

Four months before admission edema appeared and albuminuria was found. One month later hypertension was found.

On admission the urine sediment contained some erythrocytes, leucocytes, and erythrocyte casts, and many hyaline and granular casts. The eyegrounds show papillary edema and a few spots of exudate. The heart was somewhat enlarged. Blood urea nitrogen 30 mgm. per cent. Plasma cholesterol 715 mgm. per cent.

During observation the urine sediment did not change significantly except for the number of erythrocytes present. Twenty-six months after admission blood urea nitrogen was 160 mgm. per cent and creatinine 5.7 mgm. per cent. Thirty-four months after admission blood urea nitrogen was 340 mgm. per cent and creatinine 20.3 mgm. per cent.

Autopsy. Autopsy. No. 215/1924. Autopsy commenced 13 hours after death.

Death in uremia. Cardiac hypertrophy. Small fresh areas of bronchopneumonia. Serous fibrinous pericarditis. Serous fibrinous adhesive pleurisy. Ascites. Arteriosclerosis of the spleen. Moderate atheromatosis of the aortic cusp of the mitral valve, and of the coronary arteries. Advanced atherosclerosis of the aorta.

Each kidney weighed 125 grams. The capsule stripped very readily. The surface was pale grayish in color and finely and coarsely granulated. Hemorrhages have not been recorded. The cortex was much narrowed and pale yellowish in color. The differentiation of cortex and medulla was obvious.

Microscopic examination. All glomeruli are more or less severely diseased (fig. 7). They vary in size and are often very large. Mostly they are *very rich in nuclei* (fig. 8), partly with increased numbers of endothelial cells, partly with accumulation of polymorphonuclear leucocytes. Frequently the tufts are thickened by protoplasmatic or hyaline masses so that the lumina can no more be recognized. Most glomeruli are poor in erythrocytes. But single glomeruli or loops are dilated with blood. Single glomeruli are necrotic. Frequently, the glomeruli contain much fat which in part is doubly refractive. The capsular spaces occasionally contain blood. Some glomeruli are adherent to the capsules. The capsules are mostly thickened by masses stained reddish yellow by Van Gieson. The capsular epithelial cells are increased in number in some instances. Occasionally one finds thick crescents rich in nuclei. Many glomeruli are entirely hyalinized (fig. 7).

The parenchyma is penetrated by many scars which are rich in collagenous connective tissue and capillaries dilated with blood. Round cell infiltrations are frequent. Interstitial xanthoma-like cells have not been found. The tubules of these regions are in all stages of atrophy and degeneration up to entire disappearance. Part of them have undergone fatty degeneration. The better preserved tubules are dilated in part and contain coagulated protein, cellular detritus, some casts and desquamated epithelial cells. In some places they contain also some polymorphonuclear leucocytes. Part of their epithelial cells contain fat which may be doubly refractive. Hyaline-droplet degeneration has not been found.

Some arterioli show a distinct endarteritis. Frequently they contain fat. The larger arteries show a moderate intimal hyperplasia. The medulla contains scars rich in collagenous connective tissue and capillaries dilated with blood. The medullary tubules are often atrophic or degenerated and filled by an exudate similar to that of the cortex.

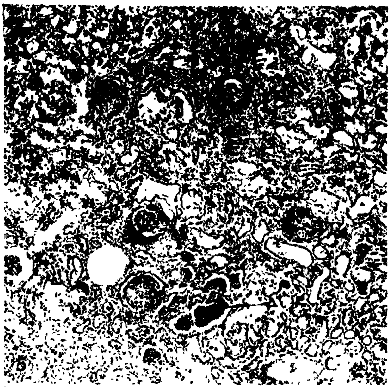
Anatomical diagnosis. Secondary contracted kidneys. Chronic glomerulonephritis.

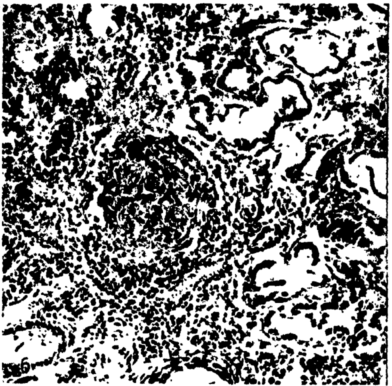
Case 35. Hospital No. 5409. M. K., female, 14 years. Height 149.5 cm. *Hemorrhagic Bright's disease.* Active chronic + pneumonia → terminal + empyema → exitus.

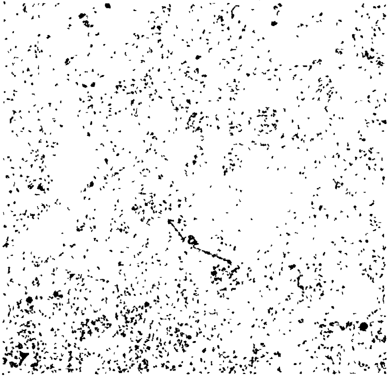
When 12 years old she had a severe cold followed by albuminuria, edema and ascites. After 6 weeks these symptoms disappeared but returned one month before admission after another cold.

On admission the urine sediment contained some erythrocytes, a few leucocytes and some hyaline and granular casts. The eyegrounds were normal, also the heart size. Wassermann reaction was negative. Blood urea nitrogen 27 mgm. per cent. Blood creatinine 1.30 mgm. per cent.

Three months after admission the patient had atypical pneumonia followed by empyema, both caused by hemolytic streptococci. Thoracotomy was performed. After that the amount of casts increased and the blood pressure rose to about 140/105. During the last month blood urea nitrogen rose to 178 mgm. per cent and bood creatinine to 8.0 mgm. per cent. Death occurred from emaciation and cardiac weakness. No autopsy.







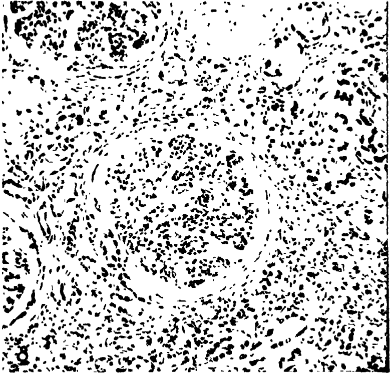


Fig. 5. Case 23. Anatomical diagnosis: subacute glomerulonephritis. Clinical diagnosis: terminal hemorrhagic nephritis developing directly from acute.

The tufts of the glomeruli are clotted with hyaline masses. The covering cells of the tufts as well as the parietal epithelial cells are greatly increased in number forming crescents. The tubules are degenerated and, in a few places, contain polymorphonuclear leucocytes (*a*). Zenkers, iron-hematoxylin-eosin. ×60.

Fig. 6. Case 23. For diagnosis see figure 5.

The tuft of the glomerulus is clotted with hyaline masses and empty of blood. The endothelial cells are necrotic in part. The glomerular space is filled by a large crescent consisting of granulation tissue. The interstitial tissue is infiltrated by round cells. Zenkers, iron-hematoxylin-eosin. ×200.

Fig. 7. Case 34. Anatomical diagnosis: chronic glomerulonephritis. Clinical diagnosis: terminal hemorrhagic nephritis.

Some glomeruli are enlarged and rich in endothelial cells and polymorphonuclear leucocytes. Some of the tufts are clotted and, in a few places adherent to the capsules (*a*). Other glomeruli are entirely hyalinized. The tubules are in all stages of atrophy and degeneration. The interstitial tissue is thickened. Zenkers, iron-hematoxylin-eosin. ×60.

Fig. 8. Case 34. For diagnosis see figure 7.

The glomerulus is enlarged and rich in endothelial cells and polymorphonuclear leucocytes. The loops are clotted and, in one place adherent to the capsule. Zenkers, iron-hematoxylin-eosin. ×200.

Case 36. Hospital No. 5178. R. N., male, 38 years. *Hemorrhagic Bright's disease.* Active chronic → terminal → exitus.

Nine months before admission edema appeared and hypertension, albuminuria and hematuria were found.

On admission the urine sediment contained a few erythrocytes and some casts. The eyegrounds were normal. Chronic tonsillitis was found. The heart was slightly enlarged. Blood urea nitrogen 45 mgm. per cent. Blood creatinine 1.71 mgm. per cent. Basal metabolic rate −4.6 per cent.

Five months after admission tonsillectomy was performed. Four months later retinal hemorrhages and papillary edema and hypertension appeared. The urine sediment contained many hyaline, granular, and erythrocyte casts and a few doubly refractive bodies. Blood urea nitrogen was 53 mgm. per cent and blood creatinine 2.33 mgm. per cent.

During the next 4 months exudative albuminuric retinitis set in. Thirteen months after admission the blood pressure was 216/134, blood urea nitrogen 86 mgm. per cent and blood creatinine 4.29 mgm. per cent. Plasma albumin had now increased to 2.66 per cent. The last 3 months before death blood urea nitrogen rose to a maximum of 119 mgm. per cent, blood creatinine to 9.38 mgm. per cent. The urine sediment contained many broad granular renal failure casts (Addis, 1925). The death was cardiac in type. The autopsy showed cardiac hypertrophy, terminal hemorrhagic Bright's disease, and congestion of the organs.

Case 37. Hospital No. 5763. G. B., male, 56 years. *Hemorrhagic Bright's disease.* Chronic active → terminal → exitus.

Three months before admission slight edema appeared and the urine was found to contain a trace of albumin and a few hyaline cases. At the same time the systolic blood pressure was found to be 190 mm. Blood pressure determinations 8, 7 and ¾ years before admission had shown normal figures.

On admission the urine sediment contained many erythrocytes and some leucocytes and hyaline, granular and erythrocyte casts. The heart size was normal. Blood urea nitrogen 27 mgm. per cent. Blood creatinine 2.10 mgm. per cent.

During observation the number of granular casts was increased somewhat. One week before death blood urea nitrogen was 300 mgm. per cent. Death occurred in uremia.

Autopsy. Autopsy No. 293/1928. Autopsy commenced 22 hours after death. Death in uremia. Uremic hemorrhages in the intestine. Cardiac hypertrophy. Fresh bronchitis with small bronchopneumonic areas. Serofibrinous pleurisy. Serous pericarditis. Arteriosclerosis of the spleen. Moderate atheromatosis of the aortic cusp of the mitral valve, of the coronary arteries and of the aorta.

The right kidney weighed 70, the left 60 grams. The capsule stripped with difficulty. The kidneys were firm. Their surfaces were grayish in color and finely and coarsely granulated. Blood dots were not seen. The cortex was much narrowed and yellowish gray in color. It was fairly well marked off from the medulla which was striped brown and yellowish.

Microscopic examination. Nearly all glomeruli are more or less altered (fig. 9). One-third of them are entirely hyalinized. Most of the rest are of usual size, but part are enlarged. Most of *their tufts are much clotted* and can no longer be differentiated distinctly (fig. 10). Most of them contain few or no erythrocytes. Frequently, they contain increased numbers of nuclei. Necroses of the loops are rare. In a few glomeruli one finds hemorrhages. The glomeruli are frequently adherent to the capsules. These are frequently thickened by masses stained red by Van Gieson. In a few places one sees proliferations of the parietal epithelial cells. In some instances one sees even crescents, some of which show spaces filled with blood between the layers. But these changes are insignificant in contrast to the intracapillary ones. Occasionally, the glomeruli contain a little fat.

The parenchyma of the kidneys is much destroyed and penetrated by scars between which preserved and dilated tubules stand out like islands. The tubules are found in all stages of atrophy and degeneration. Their epithelial cells are mostly flattened and frequently show irregular fatty degeneration. Hyaline-droplet degeneration cannot be found. In the lumina of the tubules one sees some desquamated epithelial cells, a large amount of coagulated protein, and in a few places also polymorphonuclear leucocytes.

The interstitial tissue is often enlarged and rich in collagenous connective tissue and capillaries dilated with blood. In some places it contains infiltrations of round cells. Interstitial lipoid cells cannot be found. The arterioli are mostly well preserved. A few show endarteritis. A few also are hyalinized and contain fat. The larger arteries show an intimal hyperplasia.

Anatomical diagnosis. Secondary contracted kidneys. Chronic glomerulonephritis. Secondary arteriosclerosis.

Case 38. Hospital No. 5340. E. S., female, 31 years. *Hemorrhagic Bright's disease.* Terminal → exitus.

The patient has always been subject to sore throat. Eleven years before admission she had acute nephritis of one month's duration. Three years later edema occurred for the first time; it persisted most of the time until a few months before admission. The last year headache and dyspnoea appeared.

On admission the urine sediment contained a few erythrocytes, leucocytes and hyaline casts. The eyegrounds showed fresh hemorrhages and papillary edema. The heart was increased in size. Wassermann reaction was negative. Blood urea nitrogen 40 mgm. per cent. Blood creatinine 3.31 mgm. per cent. Basal metabolic rate + 9.7 per cent.

Eleven months after admission the casts had practically disappeared from the urine, the blood urea nitrogen was 85 mgm. per cent and blood creatinine 4.81 mgm. per cent.

Twenty-four months after admission the blood urea nitrogen was 3.4 mgm. per cent and blood creatinine 16.35 mgm. per cent. Death occurred in uremia.

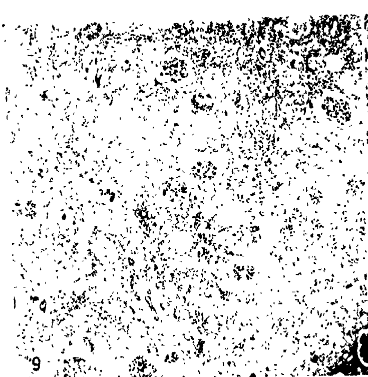
Autopsy. Autopsy No. 277/1927. Autopsy commenced 1½ hours after death. Death in uremia. Uremic hemorrhages in the intestine. Cardiac hypertrophy. Fresh bronchitis and obliterative bronchiolitis. Fresh serofibrinous adherent pleurisy of the right cavity. Fresh serofibrinous pericarditis. Fresh perisplenitis and perihepatitis. Advanced arteriosclerosis of the spleen. Advanced atherosclerosis of the abdominal aorta, and of the coronary arteries Moderate atheromatosis of the thoracic aorta and of the aortic cusp of the mitral valve.

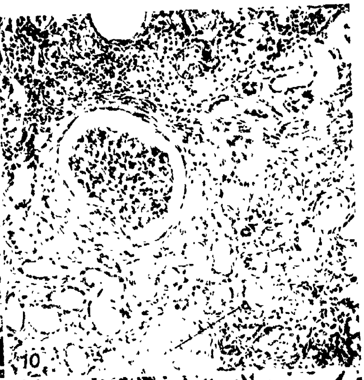
The right kidney weighed 55, the left 65 grams. The capsule stripped with difficulty. Both kidneys were firm. Their surfaces were very finely granulated and grayish in color. Small hemorrhages were numerous. The cortex was much narrowed, yellowish gray in color and contained fine blood dots. It was not well marked off from the medulla.

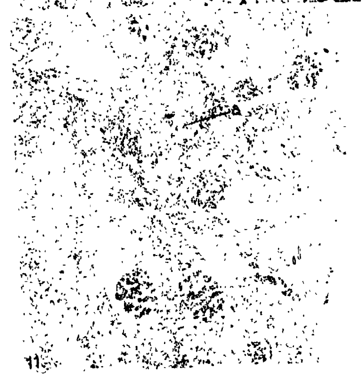
Microscopic examination. All glomeruli are more or less severely damaged (fig. 11). They are of varying, frequently enormous, size and are in all stages of hyalinization up to entire hyaline change. Mostly they are *very rich in nuclei* (fig. 12), partly of proliferated endothelial cells and partly of polymorphonuclear leucocytes which are much increased in number. Their tufts are filled with hyaline or protoplasmatic masses. Most of them are poor in blood. But, in some instances, they contain capillaries well filled with erythrocytes. Some glomeruli are necrotic and contain blood within their spaces. Frequently, the glomeruli contain fat, part of which is doubly refractive. In the glomerular spaces one sees also coagulated protein. The glomeruli are mostly adherent to the capsules. The parietal epithelial cells are partly intact. In other places they are increased in number (fig. 12). Frequently one sees also crescent formations, but these changes are insignificant in contrast to the intracapillary changes. Many glomeruli are entirely hyalinized.

The parenchyma is penetrated by many scars which are rich in collagenous connective tissue and contain many round cell infiltrations. They are rich in capillaries dilated with blood, and contain a fair number of calcified areas and chains of xanthoma-like lipoid cells (fig. 13). Occasionally one sees polymorphonuclear leucocytes entering the tubules. The tubules of these regions are in all stages of atrophy and degeneration up to entire disappearance. Their epithelial cells contain often much fat, part of which is doubly refractive. Between the scars small islands of better preserved tubules are seen, the lumina of which contain coagulated protein, some desquamated epithelial cells, some casts, and occasionally also polymorphonuclear leucocytes. The epithelial cells of the ascending loops are in places swollen (fig. 11) and contain much fat. Hyaline-droplet degeneration is rare and insignificant.

The arterioli show often an endarteritis, partly of high degree (fig. 11), and occasionally necroses of their walls. Some arterioli are hyalinized and infiltrated with fat. The larger arteries show an advanced intimal hyperplasia and in a few places also calcification. Also the medulla contains many scars rich in round cell infiltrations and capillaries dilated with blood.







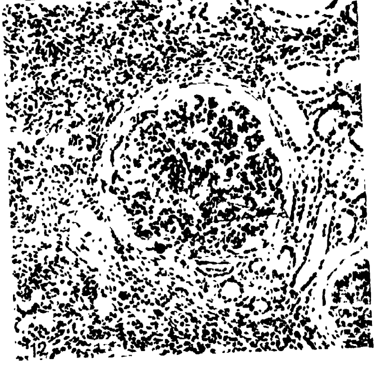


FIG. 9. Case 37. Anatomical diagnosis: chronic glomerulonephritis. Clinical diagnosis: terminal hemorrhagic nephritis.

The loops of all glomeruli are clotted and can no longer be differentiated. Their nuclei are increased in number. Some glomeruli are entirely hyalinized. The tubules are in all stages of atrophy and degeneration. The interstitial tissue is rich in scars. Zenkers, iron-hematoxylin-eosin. ×60.

FIG. 10. Case 37. For diagnosis see figure 9.

The loops of the glomerulus are clotted with hyaline masses and can no longer be differentiated. They do not contain blood. The capsule is thickened. In (*a*) a hyalinized glomerulus. Zenkers, iron-hematoxylin-eosin. ×200.

FIG. 11. Case 38. Anatomical diagnosis: chronic glomerulonephritis. Clinical diagnosis: terminal hemorrhagic nephritis.

The glomeruli are greatly enlarged and rich in endothelial cells and polymorphonuclear leucocytes. Their tufts are clotted with hyaline and protoplasmic masses and frequently adherent to the capsules. In the glomerular spaces one sees some desquamated epithelial cells. Some glomeruli are entirely hyalinized. The parenchyma contains scars, the tubules of which are in all stages of atrophy and degeneration. Between the scars one sees islands of enlarged tubules the epithelial cells of which have undergone fatty degeneration. The arterioli show a marked endarteritis (*a*). Mueller-formalin fixation, iron-hematoxylin-eosin. ×60.

FIG. 12. Case 38. For diagnosis see figure 11.

The glomerulus is enlarged and very rich in endothelial cells and polymorphonuclear leucocytes. The loops are clotted in places and adherent to the capsule. The parietal epithelial cells are increased in number. In (*a*) necrosis of a loop. The surrounding connective tissue is rich in round cells. Mueller-formalin fixation, iron-hematoxylin-eosin. ×200.

Anatomical diagnosis. Secondary contracted kidneys. Chronic glomerulonephritis. Secondary arteriosclerosis.

Case 39. Hospital No. 6215. H. B., male, 32 years. *Hemorrhagic Bright's disease.* Terminal, following 7 years latent → exitus.

The patient had had hematuria following exposure in trenches during the war, 10 years before admission. He had had edema at that time which persisted for five months. He was then told that there was a quantity of albumin in his urine. The patient had been symptom free during the following seven years. At the end of that period, or 3 years before admission to hospital, he had felt weak. He was told then that he had high blood pressure. Symptoms of headache, nocturia, and blurring of vision soon followed. Five weeks before admission hemorrhage from the gums had occurred, also a generalized body itch.

On admission to hospital, the patient was in uremia. Physical examination revealed signs of valvular disease of the heart, and cardiac enlargement. The blood pressure measured 193 mm. systolic and 124 mm. diastolic. The blood urea N was 138 mgm. per cent, and the plasma NPN 177 mgm. per cent. The phthalein excretion was but a trace in 2 hours. The urine contained 2 grams of albumin daily. Numerous red blood cells and Addis' failure casts were observed in the sediment.

During the few days that the patient was under observation, uremic symptoms became more aggravated. Bronchopneumonia developed and hastened death.

Autopsy. No. 289/1927. Autopsy commenced 17 hours after death.

Death by confluent bronchopneumonia. Cardiac hypertrophy. Serofibrinous pleurisy and pericarditis. Cartilagenous perisplenitis. Old healed endocarditis. Advanced arteriosclerosis of the spleen. Advanced atherosclerosis of the coronary arteries. Moderate atheromatosis of the aortic cusp of the mitral valve and of the aorta.

The kidneys were extremely small. The right kidney weighed 50, the left 40 grams. The capsules stripped with difficulty. Both kidneys were firm. Their surfaces were finely granulated and yellowish gray in color, showing a number of small blood dots. The cortex in each was much narrowed and similar in color to the surface. The differentiation between cortex and medulla was indistinct.

Microscopic examination. Nearly all glomeruli are more or less diseased (fig. 14). Many glomeruli are hyalinized and stained yellowish red by Van Gieson. The others vary in size and are usually poor in blood. Only a few are well filled with blood. The loops of these glomeruli are much clotted by protoplasmic and hyaline masses. Frequently they show some increase in the number of nuclei (fig. 15). Most of the glomeruli are adherent to the capsules, frequently to a large extent. Typical necroses of the glomeruli have not been seen. In the glomerular spaces one finds occasionally coagulated protein. In some instances the capsules are thickened by masses stained red by Van Gieson. *The covering cells of the tufts as well as the parietal epithelial cells are frequently increased in number* (fig. 15). Typical crescents are not seen.

The parenchyma of the kidneys is penetrated by many large scars which are rich in collagenous connective tissue and capillaries dilated with blood. Frequently they contain round cell infiltrations. The tubules of these regions are in all stages of atrophy and degeneration. Between the scars relatively few islands of better preserved tubules are seen which are dilated and filled with coagulated protein, hyaline droplets, some desquamated epithelial cells and some polymorphonuclear leucocytes. Some of their epithelial cells are flattened. Others are swollen. Occasionally one sees signs of regeneration. Many hyaline casts are seen within the tubules.

The arterioli show a high degree of endarteritis and occasionally hyalinization of their walls. The larger arteries show an advanced intimal hyperplasia which frequently has led to occlusion of the arteries. They are very rich in elastic and collagenous fibres. The tubules of the medulla are partly degenerated and are filled with a similar exudate to that of the cortex.

Anatomical diagnosis. Secondary contracted kidneys. Chronic glomerulonephritis. Secondary arteriosclerosis.

Case 40. Hospital No. 5119. R. S., female, 31 years. *Hemorrhagic Bright's disease.* Terminal → exitus.

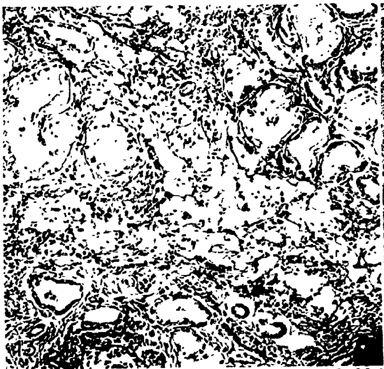
Seven years before admission the patient had diphtheria. A few months later edema appeared and albumin and casts were found in the urine. During the next 4 years she had intermittent edema, which reappeared after a severe cold 6 months before admission. The last two months nausea and vomiting were present.

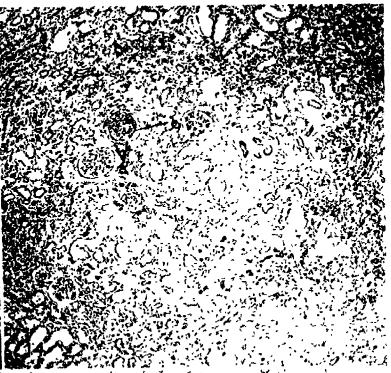
On admission gingivitis and pyorrhea alveolaris are found. The urine sediment contains a few erythrocytes and leucocytes and some hyaline and granular casts. The eyegrounds show papillary edema, hemorrhages, and white streaks of degeneration. The heart is enlarged. Blood urea nitrogen 188 mgm. per cent. Blood creatinine 4.9 mgm. per cent. Plasma cholesterol 375 mgm. per cent. Basal metabolic rate −21.4 per cent.

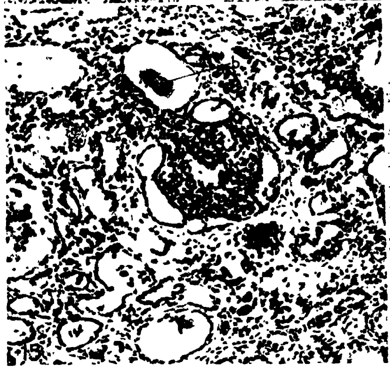
During observation the patient complained of headache and itching of the skin. The blood pressure rose to 190/120 and the blood creatinine to 9.10 mgm. per cent. Six months after admission the patient died at home with a hemiparesis. No autopsy.

Case 41. Hospital No. 6180. M. F., female, 30 years. *Hemorrhagic Bright's disease.* Terminal → exitus.

The patient had had severe headaches 4 years before admission. She had been told her blood pressure was 170 mm. at that time, also that there was albumin in her urine. After removal of her tonsils one year later, her blood pressure had fallen to 130 mm. One year prior to admission recurrent vomiting attacks had occurred. These attacks had become more frequent during the year. Edema had started two months before admission. At no time had there been gross hematuria or edema.







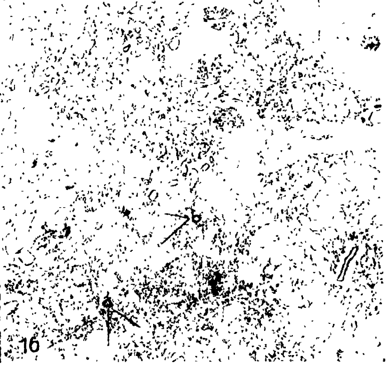


FIG. 13. Case 38. For diagnosis see figure 11.

The interstitial tissue contains a large nest of xanthoma-like lipoid cells. The tubules have undergone fatty degeneration. Mueller-formalin fixation, elastica-Van Gieson. ×200.

FIG. 14. Case 39. Anatomical diagnosis: chronic glomerulonephritis. Clinical diagnosis: terminal hemorrhagic nephritis.

The tufts of the glomeruli are clotted and frequently adherent to the capsules (*a*). The covering cells of the tufts are increased in number (*b*). The arterioli show an endarteritis. The tubules are atrophic and degenerated in part, and contain desquamated epithelial cells, polymorphonuclear leucocytes and coagulated protein. Zenkers, iron-hematoxylin-eosin. ×60.

FIG. 15. Case 39. For diagnosis see figure 14.

The loops of the glomerulus are clotted and frequently adherent to the capsule. The endothelial cells of the glomerulus, the covering cells of the loops, and the parietal epithelial cells are increased in number. In (*a*) marked endarteritis of the afferent arteriolus. In the tubules (*b*) polymorphonuclear leucocytes and cellular detritus. Mueller-formalin fixation, iron-hematoxylin-eosin. ×200.

FIG. 16. Case 41. Anatomical diagnosis: combined chronic glomerulonephritis, arterial and arteriolar sclerosis, and pyelonephritis. Clinical diagnosis: terminal hemorrhagic nephritis, plus arteriosclerotic nephritis.

The glomerular spaces are much dilated. Many glomeruli are hyalinized (*a*). The arteries show an advanced degree of arteriosclerosis (*b*). Some of the tubules are dilated and contain casts and some cellular detritus. Mueller-formalin fixation, iron-hematoxylin-eosin. ×60.

On admission to hospital there was cardiac enlargement but no signs of valvular disease. Both kidneys were palpable. The urine showed a quantity of albumin. The urinary sediment contained numerous red blood cells, no casts were seen. The blood urea N was 44 mgm. per cent, creatinine 4.5 mgm. per cent. NPN was 78 mgm. per cent, the plasma cholesterol 300 mgm. per cent. The 2-hour return of phthalein was 21 per cent of the amount intravenously injected.

During the 7 months that the patient was under observation, the blood urea N increased from 44 mgm. to 128 mgm. per cent, and the creatinine to 9.2 mgm. per cent. The phthalein excretion fell gradually to 5 per cent return of the dye in 2 hours. During the last 4 months the patient vomited continually and often became semi-comatose. Hemorrhages appeared in the fundi, and symptoms of increasing heart failure occurred. The patient died in uremic coma.

Autopsy. Autopsy No. 303/1928. Autopsy commenced 3 hours after death. Uremic hemorrhages in the intestine. Cardiac hypertrophy. Small areas of bronchopneumonia. Serous pericarditis. Advanced arteriosclerosis of the spleen. Advanced atherosclerosis of the coronary arteries. Moderate atheromatosis of the aortic cusp of the mitral valve and of the aorta.

Each kidney weighed about 70 grams. The capsule stripped easily. Both kidneys were firm. The surface was coarsely granulated, showing brown red deepenings and yellowish elevations. The cortex was much narrowed and could no longer be differentiated from the medulla. The cut surface was yellowish and mottled red brown. In a few places of the medulla small abscesses were seen containing pale yellowish pus (Staphylococcus aureus, B. Proteus). The mucous membrane of pelvis and ureter of both sides was swollen and contained large hemorrhages. Also in the bladder a number of small hemorrhages were seen. Microscopically these organs showed the picture of a fresh hemorrhagic inflammation containing Gram positive cocci.

Microscopic examination. Nearly all arterioli are severely diseased (figs. 16 and 17). They are *rich in hyaline* and, in part, contain fat. Many arterioli show *endarteritis* of varying degree. In these vessels one finds occasional *necroses of the walls* as demonstrated by decay of the tissue and by the appearance of material stained bluish by Gram. In the larger arteries one sees an advanced degree of *intimal hyperplasia.*

A very large number of glomeruli are entirely hyalinized and many are stained red by Van Gieson. Others are stained yellowish. The remaining *glomeruli are very irregular in size and show tufts empty of blood and clotted with protoplasma and hyaline substances.* A few seem to be well preserved and filled with blood. Occasionally one sees *necroses* of the tufts (fig. 18). Some more severely changed glomeruli are adhesive to the capsules and contain fat. The glomerular spaces are much dilated (fig. 16). Frequently one sees coagulated protein in these spaces. In many instances, the capsules are thickened by masses stained red by Van Gieson.

The parenchyma of the kidneys is penetrated by diffuse scars which are rich in collagenous connective tissue and capillaries dilated with blood. Frequently they contain round cell infiltrations and occasionally calcium. The tubules of these regions are in all stages of atrophy and degeneration up to entire disappearance. Their lumina are filled with hyaline casts. The tubules of the better preserved regions are extremely dilated and contain coagulated protein, casts, some desquamated epithelial cells, and, in some places, also hemorrhages and polymorphonuclear leucocytes. The epithelial cells are flattened and some contain fat.

The medulla contains some large areas the centers of which are necrotic and filled mainly with polymorphonuclear leucocytes which are often degenerated (fig. 19). These areas are surrounded by young granulation tissue. The surrounding tissue is much changed. Its tubules are degenerated and filled by many polymorphonuclear leucocytes. The interstitial tissue, here, is edematous and infiltrated by many polymorphonuclear leucocytes. Frequently one finds hemorrhages.

Anatomical diagnosis. Combined primary and secondary contraction kidneys. Arterio- and arteriolosclerosis. Chronic glomerulonephritis. Fresh hemorrhagic purulent pyelonephritis (Staphylococcus aureus, B. Proteus).

Case 42. Hospital No. 5755. F. F., male, 31 years. *Hemorrhagic Bright's disease.* Terminal, following latent → exitus.

The patient had had gross hematuria, not accompanied by edema, 3 years prior to admission. He had been told that he had Bright's disease at that time. He had had no known infection previously. Hematuria soon subsided and he had felt quite well. Apparently the disease became latent for about 2 years. Eight months before admission, however, his feet became swollen. Four months later daily vomiting attacks had occurred. One month before admission he had become very dyspneic.

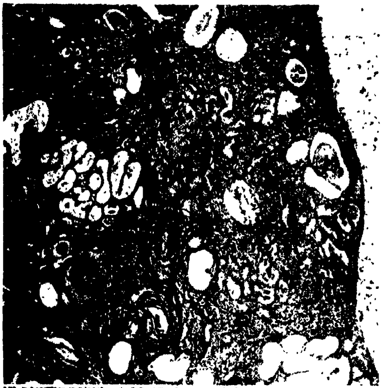
On admission the patient was in uremia. There was pitting edema of lower extremities. The blood pressure was 198 mm. systolic, and 104 mm. diastolic. The blood urea N was 175 mgm. per cent, the creatinine 15 mgm. per cent, and the hemoglobin 6.7 volumes per cent O_2 capacity. A 12-hour concentrated specimen of urine revealed an excretion of 1.0 gram of protein, 1,500,000 red blood cells, 800,000 white blood cells and epithelial cells, and 54,240 casts, all of which were of the Addis failure type.

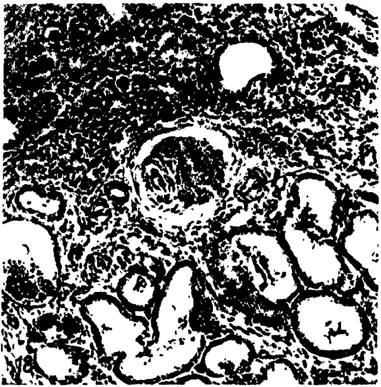
The patient was under observation but 4 days before death. During that time the blood urea N increased to 187 mgm. per cent, and the creatinine to 16.6 mgm. per cent. The patient's death was hastened by bronchopneumonia.

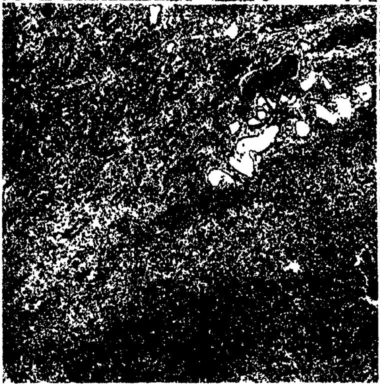
Autopsy. Autopsy No. 268/1926. Autopsy commenced 14 hours after death.

Cardiac hypertrophy. Confluent bronchopneumonia. Serofibrinous adherent pleurisy. Serofibrinous pericarditis. Ascites.

The right kidney weighed 110, the left 150 grams. The capsule stripped with difficulty. Both kidneys were doughy in consistency. The surface was slightly granulated and mottled yellowish red. Blood dots were not noted. The cortex was similar in color to the surface and well marked off from the medulla.







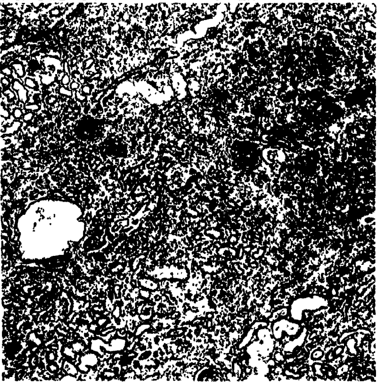


Fig. 17. Case 41. For diagnosis see figure 16.

Nearly all glomeruli (*a*) and arterioli (*b*) are hyalinized. The larger arteries show an advanced intimal hyperplasia. The parenchyma is penetrated by diffuse scars. The remaining tubules are dilated and contain hyaline casts. Mueller-formalin fixation, elastica-Van Gieson. ×60.

Fig. 18. Case 41. For diagnosis see figure 16.

Necrosis of a whole glomerulus. Endarteritis of the afferent arteriolus (*a*). The surrounding tissue is infiltrated with round cells. Mueller-formalin fixation, iron-hematoxylin-eosin. ×200.

Fig. 19. Case 41. For diagnosis see figure 16.

Purulent necrotic area (*a*) in the medulla. The surrounding tissue contains polymorphonuclear leucocytes, round cells and young fibroblasts. The tubules are filled with polymorphonuclear leucocytes (*b*). Zenkers, methylene-blue-eosin. ×200.

Fig. 20. Case 42. Anatomical diagnosis: chronic glomerulonephritis. Clinical diagnosis: terminal hemorrhagic nephritis.

The loops of the glomeruli are almost entirely clotted and adherent to the capsules to a great extent. The capsules are thickened in most instances. Some glomeruli are entirely hyalinized. The parenchyma is penetrated by diffuse scars the tubules of which are in all stages of atrophy and degeneration. Zenkers, iron-hematoxylin-eosin. ×60.

Microscopic examination. All glomeruli are severely diseased (fig. 20). Their tufts are almost entirely clotted and are adherent to the capsules to an extreme extent (fig. 21). Single loops can be recognized only with difficulty. Some of the glomeruli are empty of blood and consist of protoplasmic and hyaline masses. Others are necrotic and show hemorrhages in the glomerular spaces. In most instances these spaces are missing. In a few places one sees typical crescents (fig. 21). Most of the capsules are concentrically thickened and part are rich in nuclei. Many glomeruli are entirely hyalinized (fig. 20).

The parenchyma of the kidneys is penetrated by diffuse scars which are rich in collagenous connective tissue and capillaries dilated with blood. Frequently they contain round cell infiltrations. The tubules of these regions are in all stages of atrophy and degeneration. Between these scars some islands of better preserved tubules are seen which are dilated and filled with coagulated protein, polymorphonuclear leucocytes and some desquamated epithelial cells and casts (source of Addis' "renal failure casts"). Their epithelial cells are frequently flattened.

The arterioli show varying degrees of endarteritis. A few are hyalinized. The larger arteries show a moderate degree of intimal hyperplasia.

Anatomical diagnosis. Secondary contracted kidneys. Chronic glomerulonephritis.

Case 43. Hospital No. 4845. A. B., male, 7 years. *Hemorrhagic Bright's disease.* Terminal → exitus

About 2½ years before admission the patient had repeated tonsillitis, and albuminuria was found. This disappeared after tonsillectomy (2 years before admission), but soon reappeared and had persisted since. About 15 months before admission edema was present for 2 or 3 weeks. Three months before admission he had a period of vomiting.

On admission the urine sediment contained a few erythrocytes, leucocytes and granular casts. The eyegrounds were normal. The heart was not enlarged. Wassermann reaction negative. Blood urea nitrogen 196 mgm. per cent. Blood creatinine 5.08 mgm. per cent.

Uremia was present throughout observation. The systolic blood pressure remained about 100 mm. The diastolic gradually fell to 40. The blood urea nitrogen rose to 692 mgm. per cent and creatinine to 16.5 mgm. per cent. Death occurred in uremia. No autopsy.

Case 44. Hospital No. 4661. M. F., female, 25 years. *Hemorrhagic Bright's disease.* Terminal → exitus.

Between 5 and 1½ years before admission the patient had 3 attacks of peritonsillar abscess. After the last attack tonsillectomy was performed. Two months before admission edema gradually appeared.

On admission the urine sediment contained many erythrocytes and leucocytes, some hyaline, granular, and cellular casts, and a few erythrocyte casts. The eyegrounds showed papillary edema and exudates and a few hemorrhages. The blood pressure was 180/120. The heart enlarged. Wassermann reaction negative. Blood urea nitrogen 87 mgm. per cent.

During the first month of observation the blood pressure fell to 155/100 and remained at this level for 6 months. Fourteen months after admission it was 115/80. At this time the retinitis had also practically disappeared. Blood urea nitrogen rose slowly, however, and was 208 mgm. per cent 14 months after admission.

The patient died at home 25 months after admission.

Case 45. Hospital No. 6498. L. K., male, 39 years. *Hemorrhagic Bright's disease.* Terminal → exitus.

The patient had had a nephrectomy (right) 6 years before admission for renal calculus. Gross hematuria had occurred 4 years later, clearing up spontaneously. Hematuria again occurred the following year, lasting a few days. The left kidney pelvis had been irrigated frequently for a year preceding admission. Six weeks before admission headaches started. These were accompanied by blurring of vision and attacks of vomiting.

On admission the patient was in uremia. The blood pressure measured 165 mm. systolic, and 115 mm. diastolic. The blood urea N was 108 mgm. per cent, and the NPN of the plasma was 138 mgm. per cent. The urine sediment contained large numbers of red blood cells and broad Addis failure casts. The protein excretion in the urine was heavy. Only a faint trace of phenolsulphonephthalein was returned in 2 hours.

During the month that the patient was under observation, the blood urea N rose to 178 mgm. per cent. The blood pressure remained elevated. The day preceding death the blood creatinine was 26.4 mgm. per cent.

Autopsy. Autopsy No. 305/1928. Autopsy commenced 1 hour after death.

Death in uremia. Cardiac hypertrophy. Serous fibrinous pericarditis. Chronic cholelithiasis. Advanced atherosclerosis of the aorta.

The right kidney was missing, also the right ureter (nephrectomy). The left kidney weighed 125 grams. Its capsule stripped with difficulty. The surface was finely and coarsely granulated. Blood dots were not noted. On section the kidney showed a dilated pelvis with very little parenchyma remaining. Cortex and medulla could not be differentiated. They were grayish brown in color. The left ureter was dilated from the pelvis to the ureteral opening in the bladder.

Microscopic examination. All glomeruli are more or less diseased. Some are very large and rich in nuclei. Others are hyalinized. Their capillary loops are frequently clotted and empty of blood (fig. 22). Some contain many polymorphonuclear leucocytes. Occasionally one sees necroses and fibrin in the tufts. Most of the glomeruli are adherent to the capsules. *The covering cells of the tufts are frequently increased in number and desquamated* (fig. 22). The capsules are concentrically thickened in places by masses stained red by Van Gieson.

The parenchyma is penetrated by many scars which are rich in collagenous connective tissue and capillaries dilated with blood. Frequently one sees round cell infiltrations. The tubules of these regions are in all stages of atrophy and degeneration up to entire disappearance. Between the scars one sees islands of better preserved tubules, most of which are dilated and filled with coagulated protein, casts, relatively many desquamated epithelial cells, and some polymorphonuclear leucocytes. Their epithelial cells are swollen and degenerated. Hyaline-droplet degeneration is distinct in a few places. Occasionally one sees signs of regeneration.

The arterioli show a moderate endarteritis and, in some places a slight hyalinization. The larger arteries are in an advanced stage of intimal hyperplasia. The tubules of the medulla are dilated and filled with a similar exudate to that of the cortex.

Anatomical diagnosis. Secondary contracted kidney. Chronic glomerulonephritis. Hydronephrosis. Secondary arteriosclerosis.

Case 46. Hospital No. 5388. B. G., male, 24 years. *Hemorrhagic Bright's disease.* Terminal → exitus.

Seven months before admission the patient had sore throat and bronchitis. This was followed by edema and hematuria.

On admission chronic tonsillitis and pyorrhea alveolaris were found. There was a left dry pleurisy and some dullness over the left apex. No râles. The urine sediment contained some erythrocytes, leucocytes and hyaline and granular casts. The eyegrounds were normal. The blood pressure was about 120/80, the heart somewhat increased. Wassermann reaction negative. Blood urea nitrogen 108 mgm. per cent. Blood creatinine 5.77 mgm. per cent. Basal metabolic rate −18.7 per cent.

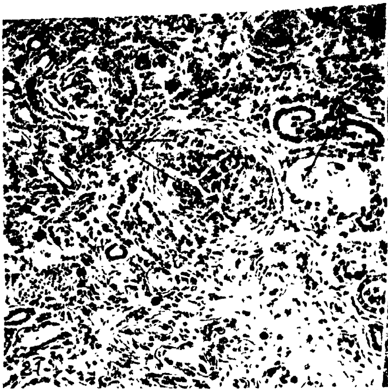
Between 3 and 12 months after admission the blood pressure rose to 146/90, blood urea nitrogen to 462 mgm. per cent and blood creatinine to 30.0 mgm. per cent. Death occurred in uremia.

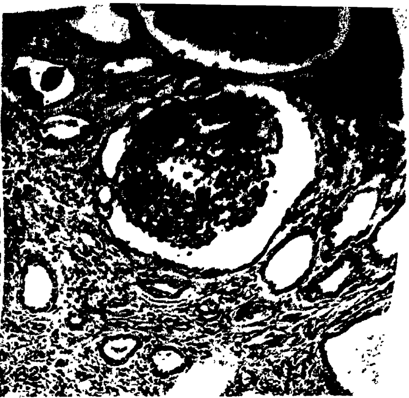
This case is of especial interest because it ran its entire observed course with practically normal blood pressure.

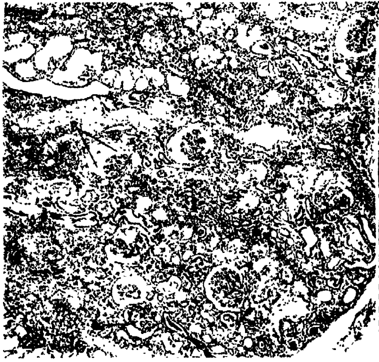
Case 47. Hospital No. 6523. E. F., male, 54 years. *Hemorrhagic Bright's disease.* Terminal + arteriosclerosis → exitus.

The patient had been told 3 years before admission that his blood pressure was high. Six months before admission he had had a collapse while working. His systolic blood pressure level at that time had been 300 mm. Dyspnea had continued to become aggravated for six months prior to his admission.

Peripheral arteries were thickened and tortuous. The blood pressure was 190/130. There was cardiac enlargement and moderate edema. The blood urea N was 45 mgm. per cent, the non-protein nitrogen 70 mgm. per cent, and the hemoglobin 17 volumes per cent O_2 capacity. The phthalein excretion was 16 per cent in 2 hours. The plasma cholesterol was 195 mgm. per cent. A concentrated specimen of urine revealed an excretion of 74,000,000 red blood cells, 3,000,000 white blood cells and epithelial cells and 970,000 casts in 12 hours.







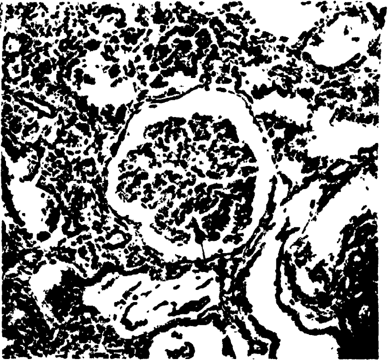


FIG. 21. Case 42. For diagnosis see figure 20.

The loops of the glomerulus are entirely clotted with protoplasmic and hyaline masses and are adherent to the capsule. The capsule is thickened. In (*a*) a crescent formation. In the tubule (*b*) one sees some polymorphonuclear leucocytes. Zenkers, iron-hematoxylin-eosin. ×200.

FIG. 22. Case 45. Anatomical diagnosis: chronic glomerulonephritis. Clinical diagnosis: terminal hemorrhagic nephritis.

The glomerulus is enlarged and rich in endothelial cells and polymorphonuclear leucocytes. The loops are clotted with hyaline and protoplasmic masses and are adherent to the capsule. The covering cells of the tufts and the parietal epithelial cells are increased in number. Zenkers, iron-hematoxylin-eosin. ×200.

FIG. 23. Case 47. Anatomical diagnosis: combined chronic glomerulonephritis and arterio- and arteriolar sclerosis. Clinical diagnosis: nephritis, terminal hemorrhagic and arteriosclerotic.

The tufts of all glomeruli are clotted with hyaline and protoplasmic masses. Some glomeruli are enlarged and adherent to the capsules (*a*). The covering cells of the tufts and the parietal epithelial cells are frequently increased in number (*b*). The capsular spaces are dilated and filled with coagulated protein and some desquamated cells (*c*). Many glomeruli are entirely hyalinized. The parenchyma is penetrated by scars which contain tubules in all stages of degeneration and atrophy. Mueller-formalin fixation, iron-hematoxylin-eosin. ×60.

FIG. 24. Case 47. For diagnosis see figure 23.

The glomerulus is enlarged and contains somewhat increased numbers of endothelial cells. The loops are clotted with protoplasmic and hyaline masses. In (*a*) one sees a necrosis of a loop. Mueller-formalin fixation, iron-hematoxylin-

Of the casts, 12 per cent were of Addis' failure type. The protein excretion in the urine was 10 grams per day. The plasma proteins were normal.

During the subsequent nine months that the patient was under observation, the blood pressure remained elevated, the blood urea N varied between 45 mgm. per cent and 200 mgm. per cent. The phthalein excretion decreased to a trace in 2 hours. The protein excretion in the urine decreased from 10 grams per day to 2 grams per day, but red blood cells and casts continued to be excreted in large numbers. Signs of increasing heart failure were present during the last five months; fluid accumulated to such an extent that it was necessary to perform a paracentesis. Throughout the last month of the patient's illness, extreme emaciation occurred. The blood urea N was 200 mgm. per cent during the last week of his illness. Death occurred after several hours in coma.

Autopsy. Autopsy No. 323/1929. Autopsy commenced 11 hours after death.

Death by cardiac and renal insufficiency. Cardiac enlargement. Ascites. Old adhesive pericarditis and pleurisy. Old healed endocarditis. Cholelithiasis. Advanced arteriosclerosis of the spleen. Advanced atherosclerosis of the aortic cusp of the mitral valve, of the coronary arteries and of the aorta.

The right kidney weighed 55, the left 65 grams. Both kidneys were firm. The capsules stripped with difficulty. The surface was finely and coarsely granulated and yellowish gray in color on a red brown background. In a few places blood dots were seen. The cortex was much narrowed and similar in color to the surface. Cortex and medulla could not well be differentiated.

Microscopic examination. Nearly all glomeruli are more or less changed and vary in size (fig. 23). A few are well filled with blood. Most of them are empty. *Their tufts are clotted with protoplasmic and hyaline masses in most instances* (fig. 24). Their nuclei appear to be increased in number. Frequently one sees partial necroses of the tufts (fig. 24). The afferent arterioli are often dilated with blood. The glomeruli are adherent to the capsules in many places. Especially in these glomeruli one sees a marked increase in the number of the covering cells of the tufts. In some places one finds typical crescents. The capsular spaces are frequently dilated and contain coagulated protein, and desquamated epithelial cells. The capsules are often thickened. Many glomeruli are entirely hyalinized and stained yellowish or red by Van Gieson. The more severely changed glomeruli contain much fat, but no lipoids.

The arteries and arterioli are severely diseased (fig. 25). The medial layer in the larger arteries is much thickened. Their intimal layer is in an advanced stage of intimal hyperplasia. *The walls of the arterioli are frequently thickened and hyalinized.* They are rich in fat in many instances. In some places they show endarteritis. Many smaller arteries are entirely closed.

The parenchyma of the kidneys is penetrated by many scars which are rich in collagenous connective tissue and capillaries dilated with blood. They contain round cell infiltrations. The tubules of these regions are in all stages of atrophy and degeneration up to entire disappearance. Between the scars islands of better preserved tubules are seen some of which are dilated. Their epithelial cells are mostly flattened. The tubules of the cortex contain numerous casts, and in some places coagulated protein, cellular detritus and desquamated epithelial cells. At one place in the cortex one sees a well limited travecular cystoma (papillary adenoma) the epithelial cells of which are equal in size and do not show karyokinetic figures. The interstitial tissue of the medulla is scarred in some places.

Anatomical diagnosis. Combined primary and secondary contracted kidneys. Chronic glomerulonephritis. Arterio- and arteriolosclerosis. Papillary adenoma.

Case 48. Hospital No. 6166. D. B., female (colored), 33 years. *Hemorrhagic Bright's disease.* Terminal → exitus.

From 6 to 3 months before admission edema of the feet was present. It reappeared one month before admission.

On admission the urine sediment contained many erythrocytes, some leucocytes, many granular and erythrocyte casts, but no doubly refractive bodies. Chronic tonsillitis was present. The eyegrounds were normal. The heart was somewhat enlarged. Wassermann reaction was negative. Blood urea nitrogen 103 mgm. per cent. Blood creatinine 7.31 mgm. per cent. Plasma cholesterol 344 mgm. per cent. Basal metabolic rate +0.3 per cent.

During observation the urine sediment did not change much except for a decrease in the number of erythrocytes. The tonsils and adenoids were removed 3 months after admission, and the patient was discharged without significant change in her outlook. Two months later she returned, with signs of cardiac failure. The standard blood urea clearance had fallen to 5 per cent of normal—about half its former value. Vomiting and twitching developed, and failure of vision, the result of an albuminuric retinitis. Death in uremia occurred 3 weeks after the second admission. Autopsy was refused.

Case 49. Hospital No. 5760. H. G., male, 47 years. *Hemorrhagic Bright's disease.* Terminal → exitus.

Six months before admission the patient for a time had frequent vomiting. The last 3 months before admission headaches were present. Five weeks before admission hypertension was found.

After admission vomiting was frequent. The urine sediment contained many erythrocytes, some leucocytes and many broad granular casts. The eyegrounds were normal. The heart was somewhat enlarged. Blood urea nitrogen 306 mgm. per cent and blood creatinine 13.04 mgm. per cent.

During treatment with intravenous salt and glucose infusions the uremic symptoms soon disappeared, the blood urea nitrogen fell to about 85 mgm. per cent and the blood creatinine to about 5 mgm. per cent. The blood pressure increased. When last seen 3½ months after admission the blood pressure had risen to about 220/115. The patient died at home in uremia. No autopsy.

Case 50. Hospital No. 5820. F. M., male, 27 years. *Hemorrhagic Bright's disease.* Terminal → exitus.

For the last 2 years before admission the patient had chronic tonsillitis. Five months before admission he caught a cold, with severe cough. During the next month paleness, dyspnea, tiredness and headaches appeared. Tonsillectomy was then performed. Two months before admission eyeground changes, proteinuria, hematuria and hypertension were found.

On admission the patient was drowsy and complained of itching and nausea. He vomited frequently, and there were occasional nose bleeds. The urine sediment contained some erythrocytes, a few leucocytes and many hyaline and granular casts. The left eyeground shows hemorrhage and white exudative spots. The heart was somewhat enlarged. Blood urea nitrogen 308 mgm. per cent. Blood creatinine 17.65 mgm. per cent. Plasma cholesterol 227 mgm. per cent.

During the 6 weeks until death the signs of uremia increased further. The blood urea nitrogen rose to a maximum of 416 mgm. per cent, the creatinine to 25.8 mgm. per cent. The blood pressure oscillated about 160/105.

ARTERIOSCLEROTIC BRIGHT'S DISEASE (ADDIS) OR NEPHROSCLEROSIS (VOLHARD AND FAHR)

General Course of the Disease

The most striking clinical features are the increase in blood pressure, and the cardiac hypertrophy, plus slight proteinuria.

As pointed out by Volhard and Fahr (1914) there are two types of the disease, the benign and the malignant.

In both forms the first clinical sign is hypertension, and the first pathological changes appear to be in the small arteries.

In the benign form the nature of the disease continues unaltered to the end, which usually does not come until many years after the first appearance of hypertension. The symptoms are almost entirely those attributable to the circulatory changes, and death eventually comes usually from circulatory rather than renal failure.

In the malignant form a rapid decrease in renal function adds itself sooner or later to the picture, and death follows, usually within some months, with symptoms of renal failure, to which are frequently added those of cardiac failure. Volhard and Fahr (1914) attributed the severe nature of these cases to the superposition of glomerular inflammation upon anteriolar disease and call it the "Kombinationsform." They consider that the end of the benign stage is marked by the development of retinitis.

The material representing arteriosclerotic disease in our series (cases 51 to 56) is scanty, particularly in relation to the incidence of this condition, which is believed to occur more frequently than other forms of renal disease. Furthermore our six cases would give one the erroneous idea that renal failure is regular in this type of disease. The reason for both these discrepancies, between our cases and the ones ordinarily met showing hypertension plus slight albuminuria, is that during the period when our observations were gathered only patients with definite renal disease were admitted to the clinic. This policy excluded the great majority of arteriosclerotic cases, because their renal signs are slight until they approach the terminal stage or the malignant development sets in.

Nevertheless these cases illustrate definite features of the disease.

Case 53 shows the transition from a benign to a malignant nephrosclerotic case. The patient was admitted with no clinical signs of renal failure, normal blood creatinine, and normal eyegrounds. Fifty per cent of injected phthalein was excreted in two hours. Only the blood urea clearance, 50 per cent of normal, indicated some decrease in renal ability. The clinical symptoms were purely those of hypertension, which had been present for eight years. Six weeks after admission protein excretion, previously only 0.3 to 0.5 gram per 23 hours, increased to 4 to 5 grams, and a rapid decrease in blood urea clearance and hemoglobin content set in. Within a month renal function had sunk to practically zero, and death occurred with symptoms combining those of uremia and cardiac failure (autopsy finding below). Cases 54 and 55 also are of the malignant type.

However, the other three cases indicate that diminished renal function can also occur ultimately in the benign, slowly progressing forms of the disease. Nos. 51, 52 and 53 showed the clinical characteristics of the benign type, and no. 51 at autopsy also showed the anatomical characteristics. Yet all three showed marked loss of renal function, and two of them, nos. 51 and 56, died in the hospital with gross nitrogen retention and the clinical symptoms of uremia, added in no. 51 to those of cardiac failure.

For explanation of the following charts see p. 495, preceding the charts of the hemorrhagic cases.

CLINICAL OBSERVATION IN CASES OF ARTERIOSCLEROTIC BRIGHT'S DISEASE (CASES 51 TO 56)

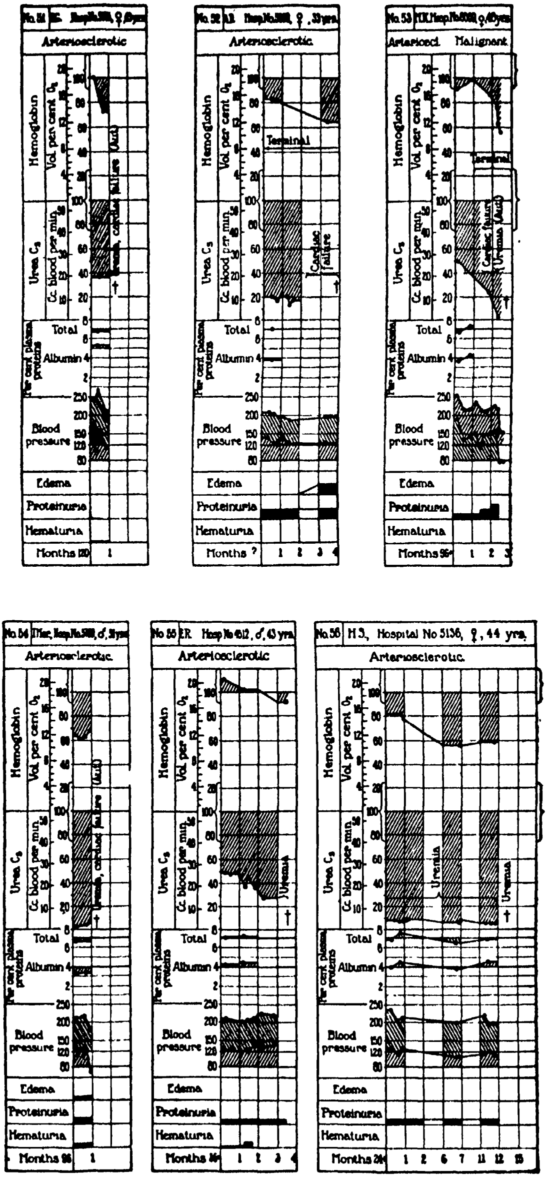
Blood urea clearance. This is low in all cases, a fact which must not be accepted as typical of any except the terminal stage of the disease, in which all of these patients were. Cases 51 and 55 died with symptoms of uremia, which, as we have stated before, are usually associated with blood urea clearances below 5 per cent of normal. Although the charts do not show it, it is practically certain that at the onset of uremia the clearances were down to this level. They were not determined, but the blood urea nitrogen values, 162, and 181 mgm. per 100 cc., were such as always accompany such clearances. In cases 53, 54 and 55, clearances in the neighborhood of 5 per cent were found, which are usual in renal deaths. It may be concluded that renal failure in all these 5 cases either was the immediate cause of death, or was sufficiently severe to have produced death in a short time, without the assistance of cardiac failure. The latter was obviously present in 4 of the 6 cases presented.

Hematuria. A microscopic hematuria is present in 3 of the 6 cases. This presumably is a terminal phenomenon. Volhard and Fahr (1914) report microscopic hematuria as rare, and Addis (1925, 1928) uses the lack of it to differentiate between the hemorrhagic and arteriosclerotic types of Bright's disease.

Blood pressure. Hypertensions, systolic as high as 200 to 250 mm., and diastolic over 120 mm., are shown by all of our cases. Again we must recall that these are terminal cases, that during most of the long course of the disease the blood pressures were undoubtedly lower, and reached their terminal heights only through increase during periods of years. Volhard and Fahr (1914) report cases with relatively moderate hypertensions, such as 170 systolic.

Plasma protein content. In all of these cases the plasma protein contents were entirely normal.

Proteinuria was present in all cases, but exceeded 1 gram per day in only two of the six. In case 53 there was a marked increase (from 0.2 to 0.5 gram per day to 4 to 5 grams) with the onset of terminal malignant symptoms. In case 52 the excretion was from 1 to 3 grams per 24 hours.


No. 51
Arteriosclerotic
No. 52
Arteriosclerotic
No. 53
Arterioscl. Malignant
No. 54
Arteriosclerotic
No 55 E.R. Hosp No 4612, ♂, 43 yrs
Arteriosclerotic
No 56 H S, Hospital No 5136, ♀, 44 yrs
Arteriosclerotic
Hemoglobin
Vol. per cent O₂
Urea C₃
Cc. blood per min.
Per cent plasma proteins
Total
Albumin
Blood pressure
Edema
Proteinuria
Hematuria
Months
Terminal
Uremia, cardiac failure (Aut.)
Cardiac failure
Cardiac failure, Uremia (Aut.)
Uremia
†

CHARTS 51–56

Edema was present in two cases, nos. 54 and 52. It was obviously of cardiac origin.

Anemia was present in five of the six cases, but to a less extensive degree than in the terminal cases of hemorrhagic nephritis. In none of the sclerotic cases did the oxygen capacity fall below 10 volumes per cent. In case 54 a rapid fall occurred from 18 volumes per cent to 10 during the terminal six weeks of the disease, when it suddenly changed from benign to malignant.

ANATOMICAL CHANGES OBSERVED IN TERMINAL NEPHROSCLEROSIS (CASES 51, 53 AND 54)

In arterio- and arteriolosclerosis we distinguish two forms which are recognized by nearly all investigators. In the one form pure arteriosclerotic lesions are outstanding, that is to say intimal hyperplasia of the larger and medium sized arteries and hyalinization and fatty degeneration of the arterioli. In the other form besides these lesions inflammatory changes are found, such as necroses and proliferations of the arterioli and glomeruli. The first form is called by Fahr (1925) benign sclerosis and coincides with the senile arteriosclerotic kidneys of Ziegler. The second form Fahr calls malignant sclerosis; it corresponds to the genuine contracted kidneys of the literature. Whereas, perhaps, most writers, like Aschoff (1921), believe that the benign and malignant forms are only two stages of one and the same disease, Fahr (1925) believes that we have to deal with two diseases. According to Fahr the benign sclerosis is primarily a pure arteriosclerotic lesion, whereas in the malignant sclerosis endarteritis and necroses are outstanding right from the beginning. He supports his theory chiefly by the finding that benign sclerosis is mainly found in old age, whereas malignant sclerosis occurs between the ages of 30 and 60.

There is no doubt that our three cases were true vascular contracted kidneys. All three showed shrunken, coarsely granulated kidneys, reddish brown in color. Histologically we found extensive hyalinization of the arterioli and glomeruli, fatty degeneration of the arterioli and extensive intimal hyperplasia of the large and medium sized arteries. A large number of glomeruli were intact. Furthermore, the cases showed advanced atherosclerosis in other regions of the body and cardiac hypertrophy.

Case 51 essentially exhibited these lesions. Therefore, we classify it with the benign sclerosis of Fahr. In addition we found increase in the number of nuclei and occasional hyaline-droplet degeneration of some glomeruli, as well as slight necroses of some arterioli and glomeruli. But these changes were only slight. They are regarded by Fahr (1925) as symptoms of decompensation of the benign sclerosis. Aschoff (1921) would call this case a transition form from the second to the third or terminal stage. It should be noticed that this case died in uremia, an outcome believed to be relatively rare in benign sclerosis.

In cases 53 and 54 we found, besides the lesions described above, extended inflammatory changes like marked endarteritis, frequent necroses of the arteriolar walls and glomeruli, occasional increases in the number of nuclei, and adhesions of the glomeruli, as well as occasional increases in the number of the glomerular epithelial cells. In case 53 there were occasional hemorrhages in the capsular spaces, in case 54 a hyaline-droplet degeneration of some glomeruli. However, most of the glomeruli were intact. Consequently, both cases represent the malignant sclerosis of Fahr.

Both cases died in uremia.

CASE HISTORY ABSTRACTS AND AUTOPSY REPORTS (ARTERIOSCLEROTIC BRIGHT'S DISEASE) (CASES 51 TO 56)

Case 51. Hospital No. 5775. H. G., female, 43 years. *Arteriosclerotic Bright's disease.* Terminal → exitus.

The patient had suffered from headaches and dyspnea on exertion for 10 years before admission. Three years before admission she had had several attacks of vertigo and nausea. These had become more frequent and more severe. One year before admission she had been told that her blood pressure was 190, and six months before admission 280 mm. During that time edema had appeared; blurring of vision had also occurred.

On admission, the heart was found to be enlarged. The blood pressure was 245 mm. systolic, and 175 mm. diastolic. The ocular fundi showed arterial tortuosity, arterio-venous compression, and fresh hemorrhages. The electrocardiogram revealed a left axis deviation. The urine sediment contained red blood cells and granular casts. The protein excretion in the urine was 2.3 grams daily. The blood urea N was 31 mgm. per cent, and the plasma NPN 47 mgm. per cent. The plasma proteins were normal. The hemoglobin was normal (18.5 volumes per cent O_2). The return of phenolsulphonephthalein in the urine in 2 hours was 43 per cent.

During the month that the patient was under observation evidence of cardiac failure appeared. The blood pressure remained elevated. The blood urea N increased to 162 mgm. per cent, and the creatinine to 9 mgm. per cent. The patient became comatose two days before death.

Autopsy. Autopsy No. 273/1926. Autopsy commenced 2 hours after death. Death in uremia. Cardiac hypertrophy. Ulcus ventriculi. Advanced Arteriosclerosis of the spleen. Advanced atherosclerosis of the coronary arteries and aorta.

The right kidney weighed 110, the left 140 grams. The capsule of each stripped easily. The surface was coarsely granulated and purplish red in color with occasional yellowish areas. Hemorrhages were not noted. The cortex was narrowed and similar in color to the surface. The differentiation between cortex and medulla was indistinct.

Microscopic examination. The arterioli and arteries of the kidneys are severely diseased (fig. 26). The arterioli are hyalinized in most places. Many are entirely closed. The larger arteries show an advanced degree of intimal hyperplasia and contain calcium. At a few places in the arterial walls one sees *slight necroses.*

Many glomeruli are intact and well filled with blood (fig. 27). Some glomeruli are a little enlarged and even show *some increase in the number of nuclei* (fig. 27). Some glomeruli contain relatively many polymorphonuclear leucocytes in their loops. Single glomeruli show necroses and *hyaline-droplet degeneration.* The glomerular spaces are wide and mostly empty. Other glomeruli are atrophic and partially hyalinized, with concentrically thickened capsules. Occasionally one sees a slight increase in the number of the parietal epithelial cells. From these glomeruli one finds all transitions to entire hyalinization. Typical signs of glomerulonephritis are nowhere seen.

The tubules are relatively well preserved. Some are enlarged. A few show hyaline-droplet degeneration. In their lumina one finds coagulated protein and some desquamated epithelial cells. The parenchyma is penetrated by scars which are rich in collagenous connective tissue and capillaries dilated with blood. They contain round cellular infiltrations. The tubules of these regions are in all stages of atrophy up to entire disappearance.

Anatomical diagnosis. Primary contractedk idneys. Arteriolo- and arteriosclerosis. Decomposed benign sclerosis of Fahr.

Case 52. Hospital No. 5868. A. D., female, 33 years. *Arteriosclerotic Bright's disease.* Terminal → exitus.

The patient suffered from headaches for several years. Eight months before admission hypertension and albuminuria were accidently found.

On admission there was no cardiac decompensation. The urine sediment contains no erythrocytes, a few leucocytes and granular casts. The eyegrounds were normal. The blood pressure was about 200/135. The heart size was markedly increased to the left. Wassermann reaction negative. Blood urea nitrogen 48 mgm. per cent. Blood creatinine 2.73 mgm. per cent.

The blood pressure did not change during treatment. Patient died at home from suddenly developed cardiac failure 4 months after admission.

Case 53. Hospital No. 6009. M. K., female, 48 years. *Arteriosclerotic Bright's disease.* Latent → terminal → exitus.

Father died of Bright's disease with severe hypertension 8 years before the patient's admission. Hypertension (190 mm.) was accidentally found.

On admission the urine sediment contained a few erythrocytes, leucocytes and hyaline casts. The eyegrounds were normal. The blood pressure was 250/190. The heart was slightly enlarged. Blood urea nitrogen was 40 mgm. per cent. Blood creatinine was 1.78 mgm. per cent. Blood urea clearance was 50 per cent normal.

Two months after admission, the urine albumin increased from 0.5 to 3.0 gram a day. Blood urea clearance had fallen to 28 per cent of normal. Then weakness and nausea appeared, but no signs of congestive heart failure. Renal failure casts (Addis, 1925) were found in the urine. Pruritus developed, then coma, and uremic twitching. Two days before death the blood urea nitrogen was 140 mgm. per cent, and the blood creatinine 9.73 mgm. per cent. The cause of this rapid development is obscure. There may have been a cardiac factor. The syndrome is like that of malignant nephrosclerosis, the "Kombinationsform" described by Volhard and Fahr.

Autopsy. Autopsy No. 282/1927. Autopsy commenced 7 hours after death.

Cardiac hypertrophy. Fresh obliterative bronchiolitis. Serous pleurisy. Serofibrinous pericarditis. Cartilagenous perisplenitis. Old healed endocarditis of the mitral valve. Advanced arteriosclerosis of the spleen. Advanced atherosclerosis of the aortic cusp of the mitral valve, of the coronary arteries, and of the aorta.

The kidneys weighed about 75 grams each. The capsule stripped with difficulty. The kidneys were firm. The surface was brownish red and the greater part was coarsely granulated. Single blood dots were seen. The cortex was narrowed and reddish and yellowish in color. It was poorly marked off from the medulla.

Microscopic examination. Nearly all arterioli are more or less diseased (fig. 28). Part of them are closed entirely. Most of them are hyalinized. Frequently they contain fat. Many arterioli show *endarteritis* of partly low and partly advanced degree. In these vessels one finds frequently *necroses of the wall* (fig. 29) as shown by granular degeneration of the tissue, pycnosis and karyokinesis of the nuclei, and by the appearance of substances in the wall which are stained bluish by Gram. In the larger arteries containing elastic tissue one sees advanced stages of intimal hyperplasia.

A large number of glomeruli are intact and well filled with blood. Others are atrophic. Many glomeruli show all transitions from beginning to entire hyalinization (fig. 28). One sees glomeruli the tufts of which are partly hyalinized. Such tufts appear to be attached to hyalinized arterioli. In other glomeruli such transitions cannot be found and the chief change is hyalinization of the loops.

Hyalinization of the capsules is seen only with advanced stages of glomerular hyalinization. In partially hyalinized glomeruli there is frequently dilatation of the afferent arterioli. Frequently *necrosis of the tufts* occurs, usually located in a single loop and very rarely affecting an entire glomerulus (fig. 30). In such glomeruli single or all loops may be dilated with blood, and very few hemorrhages may be seen in the glomerular spaces. In severely changed glomeruli one sees occasionally adhesions or a slight increase in the number of the parietal epithelial cells. In a few places also a partial increase in the number of nuclei is found. Frequently the glomeruli contain relatively large amounts of fat which often is doubly refractive.

The parenchyma of the kidneys is penetrated by many scars which are rich in collagenous connective tissue, and occasionally contain small round cell infiltrations. The scars are rich in capillaries dilated with blood. The tubules of these parts are in all stages of atrophy and degeneration up to entire disappearance. Occasionally one finds calcium in these scars. The tubules of the better preserved parts are frequently dilated. In places they show flattened epithelial cells, hyaline casts, some desquamated epithelial cells, and in a few instances also some polymorphonuclear leucocytes. The epithelial cells occasionally contain a certain amount of fat, some of which is doubly refractive. Occasionally one finds signs of regeneration. The interstitial tissue of the medulla is enlarged in some places, and rich in collagenous connective tissue.

Anatomical diagnosis. Primary contracted kidneys. Arteriolo- and arteriosclerosis. Malignant sclerosis of Fahr.

Case 54. Hospital No. 5769. J. M., male, 51 years. *Arteriosclerotic Bright's disease.* Terminal → exitus.

The patient had had two attacks of renal colic 12 and 8 years before admission. After the first attack, he had suffered from headaches. He had been told at that time that his blood pressure was 170. During the year preceding admission he had edema of the feet. Dyspnea on exertion had occurred also during that time.

On admission to hospital symptoms of cardiac failure were present, i.e., edema, respiratory distress, and cyanosis. The heart was enlarged to the left. The blood pressure measured 192 mm. systolic and 118 mm. diastolic. An electrocardiogram showed left axis deviation. The urine contained 1.5 grams albumin per liter, many failure casts, and red blood cells. The blood urea N was 72.5 mgm. per cent, the creatinine 7.7 mgm. per cent, and the plasma NPN 82 mgm. per cent. The hemoglobin was 12.2 volumes per cent O_2 capacity. The plasma albumin was 3.15 per cent, and the globulin 3.52 per cent. A trace of phthalein was excreted in two hours.

During the month that the patient was under observation, the nitrogen retention increased, the blood urea N rising to 216 mgm. per cent, and the creatinine to 16 mgm. per cent. A concentrated specimen of urine revealed by Addis's count an excretion of 19,000,000 red blood cells, 400,000 failure casts, and 1,000,000 white

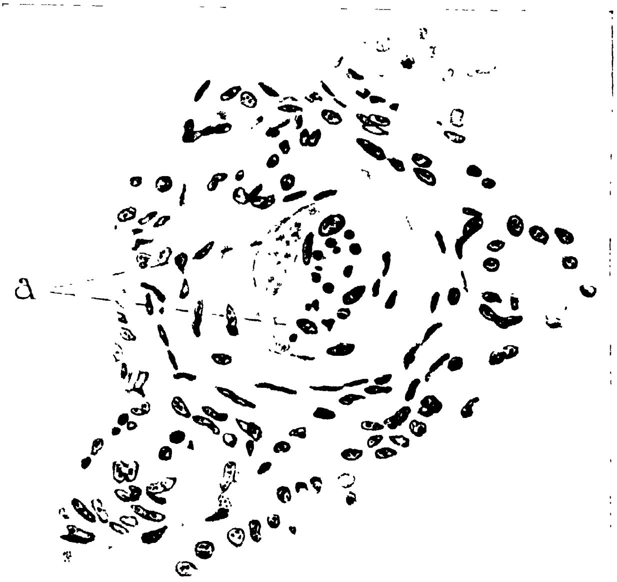
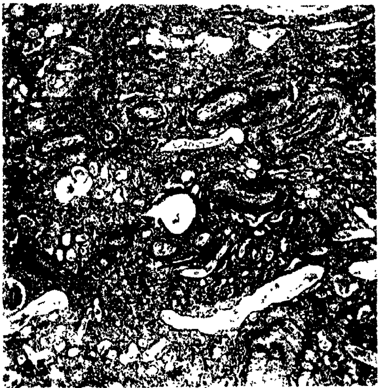
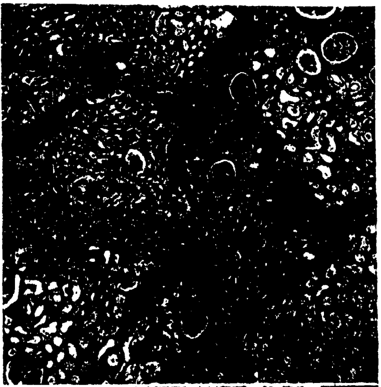
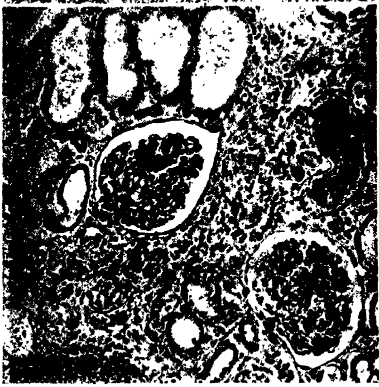


FIG. 29. Case 53. For diagnosis see figure 28.

Two arterioli with advanced endarteritis. In (*a*) necrosis of the wall with hemorrhage in the wall of the arteriole. Mueller-formalin fixation, iron-hematoxylin-eosin. ×450.







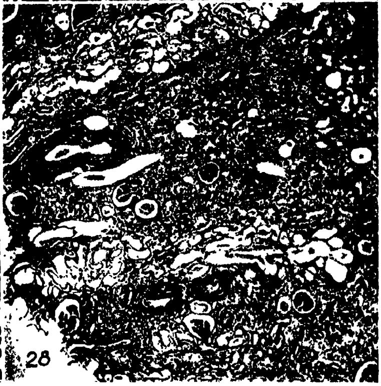


FIG. 25. Case 47. For diagnosis see figure 23.

Many glomeruli are hyalinized (*a*). The smaller and larger arteries show a high degree of intimal hyperplasia. The tubules of the scars are in all stages of atrophy and degeneration and contain many casts. Mueller-formalin fixation, elastica-Van Gieson. ×60.

FIG. 26. Case 51. Anatomical diagnosis: decomposed benign nephrosclerosis. Clinical diagnosis: arteriosclerotic Bright's disease with terminal renal and cardiac failure.

Hyalinization of the arterioli (*a*) and of some glomeruli (*b*). Advanced intimal hyperplasia of the larger arteries. Most of the glomeruli are intact. The parenchyma is penetrated by scars rich in capillaries dilated with blood (*c*). Zenkers, elastica-Van Gieson. ×60.

FIG. 27. Case 51. For diagnosis see figure 26.

(*a*) Intact glomerulus. (*b*) Glomerulus with somewhat increased numbers of endothelial cells. At the place of entrance of the arteriole beginning hyalinization. Scar formation between the glomeruli. Zenkers, iron-hematoxylin-eosin. ×200.

FIG. 28. Case 53. Anatomical diagnosis: malignant nephrosclerosis. Clinical diagnosis: arteriosclerotic Bright's disease, malignant.

Some glomeruli (*a*) and arterioli (*b*) are hyalinized. The larger arteries show an advanced intimal hyperplasia. The remaining glomeruli are intact in most instances. Mueller-formalin fixation, elastica-Van Gieson. ×60.

and epithelial cells in 12 hours. During the nine days before death, the patient lost 12 kgm. in weight. He was comatose the last 5 days.

Autopsy. Autopsy No. 271/1926.

We were permitted to do only a partial autopsy. This was performed a few hours after death.

Each kidney weighed about 100 grams. The capsule stripped easily. The surface was coarsely granulated and brownish red in color with yellowish elevations. No blood dots were found. The cortex was narrowed and similar in color to the surface. The differentiation between cortex and medulla was indistinct.

Microscopic examination. The arterioli and arteries are severly diseased (fig. 31). Some of them are closed entirely. The arterioli are hyalinized and contain much fat. Many arterioli show *endarteritis* of varying degree (fig. 32). Frequently, one finds on these vessels *necroses of the walls* (fig. 32) as indicated by granular degeneration of the tissue, pycnosis of the nuclei and hemorrhages within the walls. The larger arteries show an advanced intimal hyperplasia and are partly calcified.

Many glomeruli are entirely hyalinized (fig. 31). Others show all transitions from beginning hyalinization, starting in most places in the loops, up to entire degeneration. The remaining glomeruli are intact in most places and well filled with blood. In some glomeruli one finds *necrosis of the loops, a hyaline-droplet degeneration*, some fat and a partial increase in the number of nuclei. In severely changed glomeruli one sees occasionally adhesions and a slight increase in the number of the parietal epithelial cells.

The parenchyma is penetrated by scars which are rich in collagenous connective tissue and capillaries dilated with blood. Frequently one sees round cell infiltrations. The tubules of these regions are in all stages of atrophy and degeneration. Some of the better preserved tubules are dilated and contain coagulated protein and desquamated epithelial cells. Occasionally one sees fatty degeneration of the epithelial cells. The interstitial tissue of the medulla is enlarged in places and rich in collagenous connective tissue.

Anatomical diagnosis. Primary contracted kidneys. Arterio- and arteriolosclerosis. Malignant sclerosis of Fahr.

Case 55. Hospital No. 4512. E. R., male, 43 years. *Arteriosclerotic Bright's disease.* Terminal → exitus.

Three years before admission a systolic blood pressure of 165 was accidentally found. One year later exhaustion and headaches appeared. Seven months before admission the patient was examined with the following result; blood pressure 232/135, eyes normal, some pyorrhea alveolaris, the urine contained a trace of albumin and some hyaline, granular, and waxy casts. Two-hour phthalein excretion after intramuscular injection was 37.5 per cent. Non-protein N was 30 mgm. per cent.

On admission the urine sediment contained a few erythrocytes and leucocytes and some hyaline and granular casts. The eyegrounds showed thin arteries and a

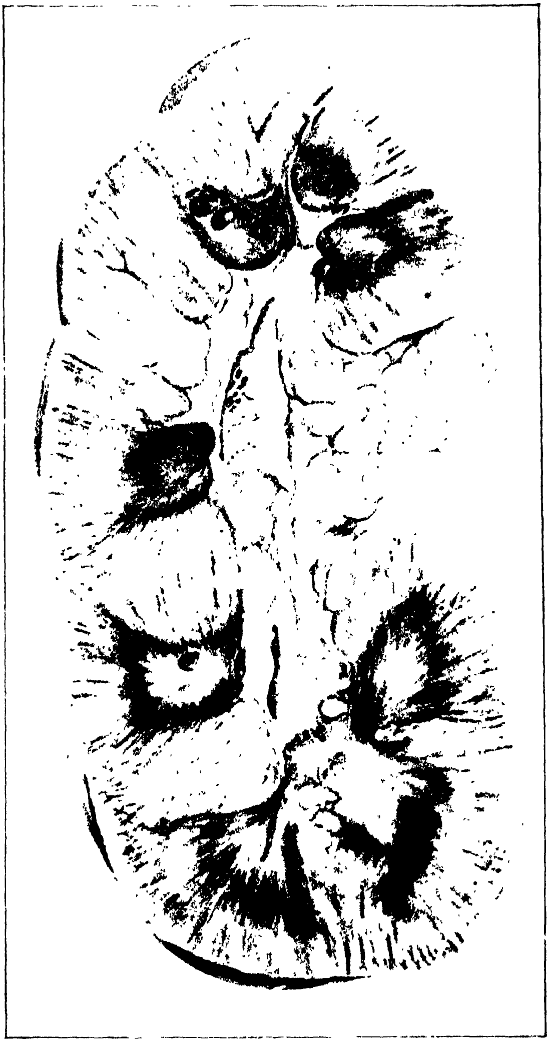
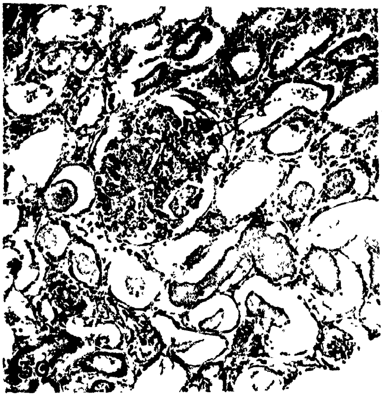
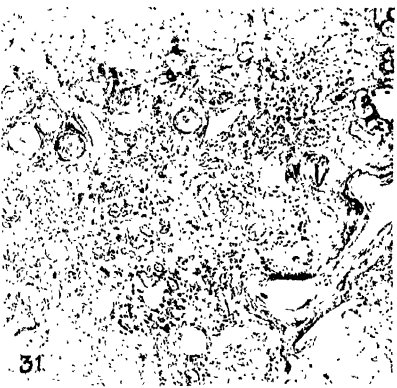
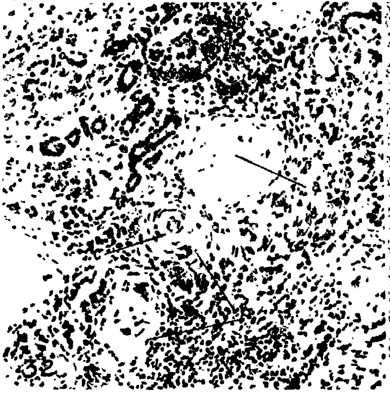


FIG. 33. Case 59. Anatomical diagnosis, lipoid nephrosis. Clinical diagnosis: chronic non-hemorrhagic degenerative Bright's disease.

Longitudinal section through the right kidney drawn after Jores fixation. Natural size.







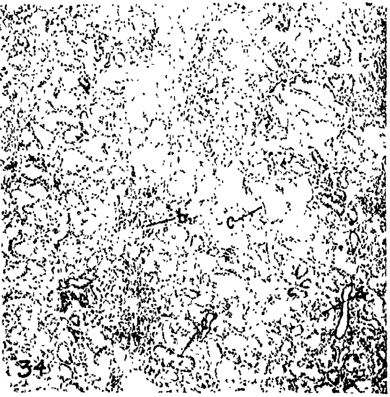


Fig. 30. Case 53. For diagnosis see figure 28.

The whole glomerulus is necrotic. Some of the loops are dilated with blood (*a*). The tubules contain many hyaline casts. Mueller-formalin fixation, iron-hematoxylin-eosin. ×200.

Fig. 31. Case 54. Anatomical diagnosis: malignant sclerosis. Clinical diagnosis: arteriosclerotic Bright's disease with terminal cardiac and renal failure.

Hyalinization of many glomeruli. Advanced intimal hyperplasia of the smaller and larger arteries. The remaining glomeruli are intact. The parenchyma is penetrated by scars which contain capillaries dilated with blood and tubules in all stages of atrophy and degeneration. Round-cell infiltration (*a*). Mueller-formalin fixation, elastica-Van Gieson. ×60.

Fig. 32. Case 54. For diagnosis see figure 31.

(*a*) Hyalinized glomerulus. (*b*) Arterioli showing an advanced endarteritis and necrosis of their walls. (*c*) The surrounding tissue is scarred and contains a round cell infiltration. Mueller-formalin fixation, iron-hematoxylin-eosin. ×200.

Fig. 34. Case 59. For diagnosis see figure 33.

Enlarged convoluted tubules with fatty, vacuolic or hyaline-droplet degenerated epithelial cells. Distinct striped scars with infiltrations of round cells. In (*a*) one sees an intact glomerulus, in (*b*) a small intact artery, in (*c*) regeneration of epithelial cells. Mueller-formalin fixation, iron-hematoxylin-eosin. ×60.

few small hemorrhages. The blood pressure was about 210/125. The heart was enlarged. Wassermann reaction negative. Blood urea nitrogen 49 mgm. per cent.

During observation the blood urea nitrogen rose to 111 mgm. per cent. One month after his discharge the patient was admitted to another hospital. He was then in uremia and died a few days later. The blood urea nitrogen was then 181 mgm. per cent. No autopsy.

Case 56. Hospital No. 5136. H. S., female, 44 years. *Arteriosclerotic Bright's disease.* Terminal → exitus.

Two years before admission nocturia occurred. One year later occipital headaches and dyspnea on exertion appeared. For the last 2 weeks before admission she had vomiting in the morning.

On admission the urine sediment contained nothing pathologic. The eyegrounds were normal. The blood pressure was 238/130 and the heart was enlarged. Blood urea nitrogen 174 mgm. per cent. Blood creatinine 8.70 mgm. per cent.

During observation the urine sediment showed only occasional leucocytes and single hyaline casts. The blood pressure was about 210/115. Blood urea nitrogen rose gradually to 297 mgm. per cent and creatinine to 11 mgm. per cent. Throughout observation she had headaches, and nausea with occasional vomiting. Fifteen months after admission, when at home, she contracted pneumonia and died in coma with convulsions. No autopsy.

NON-HEMORRHAGIC DEGENERATIVE BRIGHT'S DISEASE (ADDIS) OR NEPHROSIS (VOLHARD AND FAHR)

General Course of The Disease

Müller (1905) and Volhard and Fahr (1914) have pointed out the existence of a type of Bright's disease distinguished by heavy proteinuria, tendency to edema formation, usually massive, and by degenerative rather than inflammatory changes in the kidneys. The disease appears to accompany or follow a heterogeneous set of intoxications and infections, frequent among which are pregnancy, toxemia, osteomyelitis, syphilis, and tuberculosis. In many cases there is no obvious etiology: the edema begins insidiously and without previously noted illness or intoxication. Addis (1925) calls these cases "cryptic" and Volhard and Fahr term them "genuine."

The outstanding constant abnormalities in the blood are a deficit of plasma albumin, which is more often than not less than half the normal 4 per cent concentration, and an increase of the fat and cholesterol contents. The work of Starling, Epstein, Govaerts, and Schade and Claussen, and Moore and Van Slyke, quoted above in the discussion of the significance of plasma protein values, has left small room to doubt that the plasma albumin deficit is the predisposing factor for the development of edema.

The quantitative relationships between the tendency to edema formation, the deficit of albumin and total protein in the plasma, and the decrease in plasma specific gravity have been outlined in the above mentioned discussion.

The lack of 4 abnormalities that are common in hemorrhagic nephritis has been used to differentiate nephrosis from the former. These are hematuria, urea retention, hypertension, and anemia, all of which are common in chronic hemorrhagic nephritis, and may be entirely absent in nephrosis. However, of the four, *hematuria and hypertension* are the only ones we have found *uniformly absent* in our series of nephrosis cases.

With regard to nitrogen retention and ultimate uremia, data in the literature differ. Volhard and Fahr (1914) observed nitrogen retention rarely, and apparently considered it unlikely to become serious. They had not seen a death attributable primarily to renal failure. Addis on the other hand (1925) remarks: "The disease may pass through active and latent stages to complete recovery, or may gradually progress to a termination in uremia." Of our 10 cases two have progressed to final uremia with urea retention (nos. 65 and 66). Furthermore it seems safe to predict that such an outcome is probable in two others, cases 61 and 64, which for two years have shown in general a downwards tendency in their renal function, as measured by the blood urea clearance. Volhard and Fahr found part of the glomeruli altered in cases which they autopsied. It is not strange that the damage should in time proceed far enough to result in uremia. Our data indicate that the process may be slow. On the other hand, in case 66 the entire apparent duration of the disease to uremia was only a year and a half. The absence of evidence of inflammatory processes in this case was demonstrated by autopsy.

Anemia likewise is by no means uniformly absent in nephrosis. Considering the malnutrition that appears to be inherent in the disease, and the frequency with which the patients suffer from obstinate infections, the occurrence of anemia is not surprising, even when it can not be attributed to the effect of toxic retention. The effect of intercurrent infections appears to be illustrated by case 61. The hemoglobin, from a full normal value, fell after septicemia and pleurisy succeeding one another, to 40 per cent. Thereafter it gradually rose again, reaching 80 per cent within a month and ultimately 100 per cent, despite the fact the renal disease, as indicated by blood urea clearance and albuminuria, had progressed rather than improved. Of our 10 cases, only 3 in fact consistently showed hemoglobin values above 80 per cent of Haldane's average normal standard.

In the latent form of hemorrhagic nephritis, and less frequently in the chronic active stage of hemorrhagic nephritis, hematuria and hypertension, mentioned as characteristically absent in nephrosis, may be lacking, or very slight. As mentioned in discussion of these stages of hemorrhagic nephritis, differential diagnosis from nephrosis may be difficult unless there is a history of acute hemorrhagic onset. In the latent hemorrhagic disease the plasma protein deficit and albuminuria are likely to be less marked, however, than in nephrosis; and if the primarily hemorrhagic case is watched it is likely either to make a rather rapid recovery (which is not frequent in nephrosis), or else if the disease progresses to the terminal stage, to show a return of hematuria and develop a hypertension, as in case 23a.

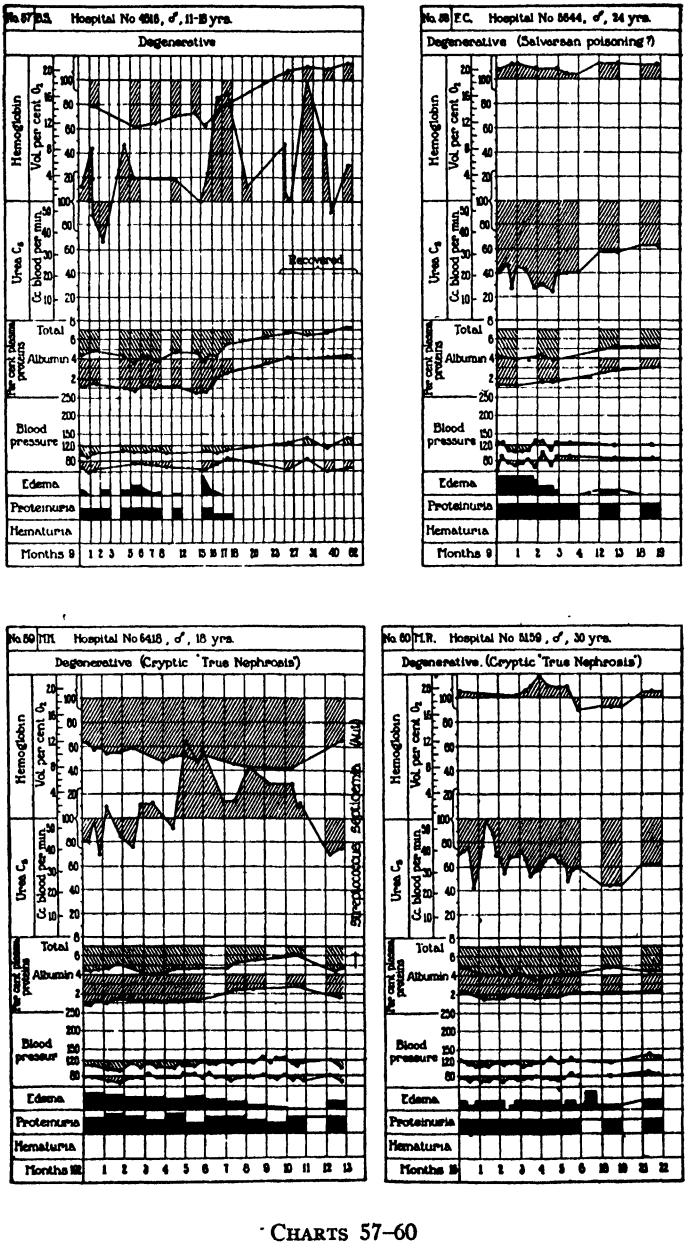
For explanation of the following charts see page 495 preceding the charts of the hemorrhagic cases.

CLINICAL OBSERVATIONS IN CASES OF DEGENERATIVE BRIGHT'S DISEASE (CASES 57 TO 66)

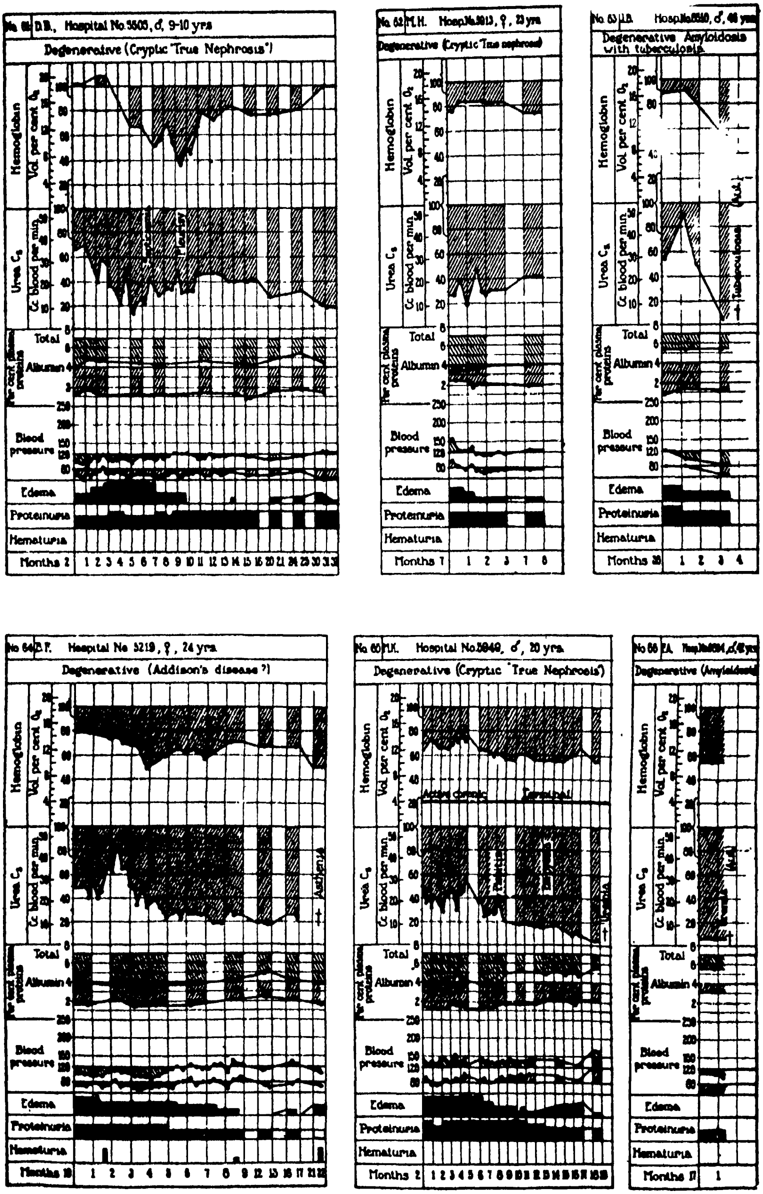
Urea excreting power. In only 2, nos. 57 and 59, of our 10 cases, was the blood urea clearance up to normal throughout the course of observation. In these two there were periods when the urea excretion was indeed hypernormal, a phenomenon elsewhere met only in cases recovering from acute hemorrhagic nephritis.

In 8 cases (nos. 58, 60, 61 and 62 to 66), there was definite fall in blood urea clearance, apparently temporary in case 58 (disease attributed to salvarsan poisoning), but showing a tendency to remain or progress in the others.

In 3 cases (nos. 63, 65 and 66) the blood urea clearance before death


No 57 B.S. Hospital No 4616, ♂, 11-15 yrs.
Degenerative
Hemoglobin
Vol. per cent O2
Urea Cs
Cc. blood per min.
Recovered
Per cent plasma proteins
Total
Albumin
Blood pressure
Edema
Proteinuria
Hematuria
Months 9 1 2 3 5 6 7 8 12 15 16 17 18 20 23 27 31 40 62
No 58 F.C. Hospital No 5644, ♂, 24 yrs.
Degenerative (Salvarsan poisoning?)
Months 9 1 2 3 4 12 13 18 19
No 59 M.M. Hospital No 6418, ♂, 18 yrs.
Degenerative (Cryptic "True Nephrosis")
Streptococcus septicemia
Months 12 1 2 3 4 5 6 7 8 9 10 11 12 13
No 60 M.R. Hospital No 5159, ♂, 30 yrs.
Degenerative. (Cryptic "True Nephrosis")
Months 15 1 2 3 4 5 6 18 19 21 22

CHARTS 57–60


No 61 D.B., Hospital No 5505, ♂, 9-10 yrs
Degenerative (Cryptic 'True Nephrosis')
No 62 M.H. Hosp.No.3013, ♀, 23 yrs
Degenerative (Cryptic 'True nephrosis')
No 63 J.B. Hosp.No.5510, ♂, 46 yrs
Degenerative Amyloidosis with tuberculosis
No 64 B.F. Hospital No 5219, ♀, 24 yrs
Degenerative (Addison's disease ?)
No 65 M.K. Hospital No.3649, ♂, 20 yrs
Degenerative (Cryptic 'True Nephrosis')
No 66 F.A.
Degenerative (Amyloidosis)
Hemoglobin
Vol. per cent O₂
Urea Cₛ
Cc. blood per min.
Per cent plasma proteins
Total
Albumin
Blood pressure
Edema
Proteinuria
Hematuria
Months
Active chronic
Terminal
Tuberculosis
Asthenia
Uremia
Pleurisy

CHARTS 61–66

fell to the uremic level, and cases 65 and 66 died typical renal deaths. Case 66 at autopsy proved to be amyloid nephrosis, with extensive glomerular damage. For no. 65 autopsy was refused.

Hematuria was consistently absent in all of our cases, except for single examinations during 2 years in case 64.

Blood pressure. There was no hypertension in any case throughout the observed course of the disease. The only exception is a single observation of 154/98 on case 62 immediately after admission, which may probably be disregarded. In case 63 with tuberculosis and asthenia the pressure was abnormally low.

Plasma protein concentration. Of all the chemical examinations in nephrosis that of the plasma protein concentration is of first interest, because the albumin deficit is so closely related to the tendency to edema which is the disabling factor of the disease.

In every one of our cases the plasma albumin content was below 2.5 per cent on admission, and total proteins were below 5.5 per cent, usually below 5.0.

The cases are arranged, as in the previous groups, in order with those of apparently most favorable clinical outcome, in so far as the disease is concerned, first in the series.

The first 3 cases are the only ones that showed definite and significant rises in total protein and albumin content during the course of observation. Of these no. 57 made an apparently complete recovery and returned to the normal activities of a boy of his age. His plasma proteins returned completely to normal level 2 years after observation was begun, and 2 and $\frac{3}{4}$ years after symptoms of the disease were first noted. Case 58 left the hospital subjectively well, and apparently on the road to ultimate recovery. Case 59, of 16 years duration, improved in 1 year from a condition of complete helplessness to one in which he could walk indoors and out and begin to renew relatively sedentary activities. During this period of improvement the plasma albumin rose from 0.9 to 2.8 per cent, passing the critical level previously discussed, and edema became relatively manageable. There was a fall in plasma albumin and some increase in edema during a period of a month's absence from the hospital. The streptococcus infection terminated a case which had begun to arouse hopes of a cure like that of no. 57.

In none of the other cases (nos. 60 to 65 inclusive) did the plasma albumin rise above 2.5 per cent, and in none of them was there a permanent disappearance of the edema.

No. 66 represents an exception among pure degenerative cases, in that the edema was never severe. Even without treatment it had increased the body weight only by a few pounds. The subjective symptoms were those of a terminal hemorrhagic case rather than those usually typical of nephrosis. In accordance with these observations there was found a uremic nitrogen retention, but only a moderate plasma protein deficit, the albumin being 3 per cent and the total 5.5. The case was diagnosed nevertheless as nephrosis because of the consistently low blood pressure and the absence of microscopic hematuria. Autopsy showed a typical amyloid nephrosis.

The relatively moderate albumin deficit is not characteristic of amyloid nephroses: they usually show deficits equal to those of lipoid nephrosis. Case 63 for example, showed the usual marked deficit. No. 66, however, demonstrates that it is possible for exceptional pure degenerative Bright's disease, at least of the amyloid type, to occur with only moderate plasma protein loss and edema.

Proteinuria was marked in all cases, the output varying from 2 grams per day to over 20 grams.

Edema was present in all cases, except no. 66, in which the plasma albumin was relatively high. Elimination of edema in cases 57, 58, 59 and 64 occurred under the combined effect of treatment and increase of plasma albumin. In cases 60, 61 and 62 edema diminished or disappeared temporarily under the influence of treatment without increase in plasma albumin.

The data regarding *anemia* have already been sufficiently discussed above.

ANATOMICAL CHANGES OBSERVED IN DEGENERATIVE BRIGHT'S DISEASE (CASES 59, 63 AND 66)

Nephroses include, according to Fahr (1925), all primary degenerative diseases of the kidneys which attack the glomeruli and the tubules, but not those which attack the vascular system. Fahr separates from the plain nephroses a group in which, besides degenerative lesions, infiltrative changes occur, which, according to him, are due to disturbances in metabolism. This group he called the definitely characterized nephroses, and classifies with them the so-called lipoid and amyloid nephroses. Aschoff (1921), on the contrary, does not believe that these two diseases are of the same nature, but ascribes amyloid nephrosis, which he calls amyloid infiltration, to nephrodystrophies, and lipoid nephrosis to inflammations of the kidneys, calling it tubular nephritis. Aschoff (1913, 1917) also objects to the term nephrosis and logically argues that this term should be replaced by "nephrodystrophia" or "nephropathia." If here, nevertheless, we use the term, nephrosis, it is because it is generally accepted by clinicians, and especially because no agreement has yet been reached concerning the nature of this disease. For similar reasons we also discuss here the amyloid and lipoid forms together, because they resemble each other in many respects, not only clinically, but also anatomically.

Lipoid nephrosis. Case 59 has been reported in detail elsewhere (Ehrich, 1929). Here we shall, however, discuss it briefly. Macroscopically, we found large white kidneys without visible blood dots. Histologically, an intense fatty and hyaline-droplet degeneration of the epithelial cells was outstanding, in comparison with which all other changes were insignificant. There was indeed marked scar formation and hyalinization of half of the glomeruli. But most of the preserved glomeruli were intact. Only in a few glomeruli increased numbers of nuclei were seen. But this increase was not out of proportion to the size of the glomeruli. In some hyalinized glomeruli there were slight proliferations of the parietal epithelial cells. All these lesions are found, according to Fahr (1918, 1922) and others, more frequently in advanced cases of lipoid nephrosis. Furthermore, if we take into consideration the clinical observations, there can be no doubt of the diagnosis "genuine lipoid nephrosis."

Of special interest was the fact, that, in spite of a duration of about 17 years, no shrinkage of the kidneys had occurred. Whereas Fahr (1925) and others believe that lipoid nephrosis leads to contracted kidneys, Aschoff (1913) and others express doubt. That amyloid nephrosis may lead to contracted kidneys is no wonder, if we take into consideration the great destruction of glomeruli, which is generally assumed to lead to contraction. However, in lipoid nephrosis, the conditions are quite different. Here, the glomeruli are much less diseased and there is no extensive destruction of them. There appears to be in the literature no case of contracted kidney which can with certainty be ascribed to pure lipoid nephrosis (Ehrich, 1929). Our case, which seems to be the only genuine one of such long duration observed, shows that prolonged lipoid nephrosis does not necessarily lead to contracted kidneys.

Amyloid nephrosis. Whereas lipoid nephrosis is only rarely observed in autopsy, amyloid nephrosis occurs frequently. Concerning its morphological course Volhard and Fahr (1914) have distinguished three stages and Fahr later on (1925) four stages, for the purpose of facilitating description.

Case 63 showed lesions characteristic of the second stage. Besides amyloid infiltration, severe degenerative changes were found. Both kidneys were very large. The glomeruli still contained many loops filled with blood. A slight scar formation was present. The changes of the tubules were essentially the same as those in lipoid nephrosis.

Case 66 we ascribed to the third stage, because a marked scar formation had already developed and because the amyloid no longer gave the iodine sulfuric acid reaction, whereas the methyl violet reaction in part was still positive. The glomeruli were greatly infiltrated with amyloid. Most of the glomeruli contained no erythrocytes. The destruction of the glomeruli and arterioli was so extensive that a renal insufficiency and uremia developed. The tubules were in the stage of advanced atrophy, whereas fatty and hyaline-droplet degeneration were insignificant.

Our cases of amyloid nephrosis were of special interest also from the etiological point of view. Whereas the amyloidosis of case 64, as so frequently occurs, was due to tuberculosis, in case 66, in spite of thorough examination, no visible cause could be found. Examination of the bones at autopsy was not permitted. But, because no clinical symptoms were present, we believe we can exclude a bone disease with certainty. Nor can the old healed endocarditis, which was found in this case, be held responsible for the amyloidosis. We have to place this case, with a number of others reported in the literature (cf. Fahr, 1925), in the group of unknown etiology.

CASE HISTORY ABSTRACTS AND AUTOPSY REPORTS. DEGENERATIVE BRIGHT'S DISEASE (CASES 57 TO 66)

Case 57. Hospital No. 4616. B. S., male, 11 years. *Degenerative Bright's disease. Progressing to cure.*

Ten months before admission the patient had sore throat. Three weeks later he had swelling of face and neck, and albuminuria was found. Spent 6 weeks in Bronxville hospital, then 4 months in bed at home. Then returned to school. Swelling of face increased. There were no constitutional symptoms nor hematuria.

On admission tonsils and adenoids were found enlarged. Heart was slightly enlarged, without valvular disease. Blood pressure was 95/70. There was swelling of face and ankles. The urine contained 4 grams of albumin per liter, a few epithelial cells, granular casts, hyaline casts, and white cells. Very few red cells were found on 2 occasions in first 3 weeks. Edema quickly cleared up. Diseased tonsils and adenoids were removed. Edema tended to recur, and was combatted with urea administration, which he continued to take at home, 3 months after admission. Returned to hospital 16 months after first admission with massive general edema. While patient was under observation catarrhal otitis media developed. At the same time the edema disappeared, and albuminuria decreased. Two months later only faint traces of albumin remained. Subsequent observations showed the urine free of albumin and cellular elements.

Case 58. Hospital No. 5644. F. O., male, 24 years. *Degenerative Bright's disease.* Syphilis.

Since the age of 18 the patient has had chronic periostitis of both tibiae. A ++++ Wassermann reaction was found, and several treatments with salvarsan and mercury were given with good effect. Eight to 9 months before admission, during such treatment, edema appeared. Four months before admission a gangrenous appendix was removed.

On admission a typical luetic tibial periostitis and signs of a previous keratitis were found. The urine sediment contained no erythrocytes, some leucocytes and double refracting bodies, and many hyaline and granular casts. The eyegrounds, blood pressure, and heart size were normal. Wassermann reaction negative. Blood urea nitrogen 45 mgm. per cent. Blood creatinine 1.71 mgm. per cent.

The plasma albumin increased to 2.96 per cent and edema gradually disappeared. Since 16 months after admission the patient had been doing a full day's work and eating an ordinary diet. The Wassermann reaction is still negative. The sediment still contains a few hyaline and granular casts.

Case 59. Hospital No. 6184. M. M., male, 17 to 18 years. *Degenerative Bright's disease of 17 years' duration* → exitus.

The patient was apparently well at birth. At age 16 months onset of present illness occurred with facial edema and albuminuria. For 14 years he was on a low protein and salt-free diet and continued to excrete large amounts of albumin. He has had recurrent attacks of edema every two years lasting 2 to 3 weeks until the present attack, which had its onset 2 years and 5 months before admission. He was treated for this with high protein diet, tonsillectomy and several tooth extractions. Owing to edema and time in bed, severe contractures of posterior muscle groups of both legs were present.

On admission he was pale, edema of extremities was pronounced; he had marked ascites, several badly decayed teeth, and distinct anemia (hemoglobin 11.6 volume per cent O_2). Cholesterol was 272 mgm. per cent, plasma albumin was only 0.9 per cent, globulin 3.39 per cent. The urine sediment contained many hyaline and granular casts, occasional red blood cells. Albumin excretion in urine averaged 6 to 9 grams per day. The blood pressure was 108/78. Renal function was slightly subnormal.

Over a course of 9 months the patient was on a high protein and salt-free diet. During this time most of the edema disappeared, and the contractions of legs were reduced to extent that he could walk unaided. The albumin rose to 2.8 per cent; globulin remained about the same level at 3.2. The plasma cholesterol fell to 187 mgm. per cent. During the entire course under observation the blood pressure remained low.

Patient was readmitted after one month because of slight increase of edema of legs and puffiness about the eyes. Subjectively well. Two weeks later the onset of general peritonitis (hemolytic streptococcus) was marked by a chill, a rise of temperature to 105°, and abdominal pain. December 2, 1928, death occurred from general peritonitis.

Autopsy. Autopsy No. 311/1928. We were only permitted to do a partial autopsy, limited to the kidneys. This was performed about 2 hours after death.

Each kidney weighed about 300 grams. The capsule stripped easily. The surface was pale yellowish in color and smooth, with indications of fine light violet shadings. Blood dots could not be found. On cross section (fig. 33) the cortex was much enlarged, measuring from 9 to 20 mm. It was pale yellowish in color and contained fine radiate light violet stripes. The cortex was fairly well marked off from the medulla. In the pelvis a few hemorrhages were seen.

Microscopic examination. The cortex contains stripes and patches of enlarged tubules (fig. 34) the character of which as convoluted tubules is still distinct in many places. In places these tubules are lined by *much swollen epithelial cells that have undergone fatty or vacuolic degeneration* (fig. 35) closing the tubules partly or entirely. In places the latter are lined by epithelial cells *far gone in hyaline-droplet degeneration.* The epithelial cells, mainly of these degenerated tubules, frequently contain pycnotic nuclei or have lost nuclei entirely. In many instances the epithelial cells are desquamated. In such places one finds signs of regeneration (fig. 36). Gaps in the epithelial layer are covered by endothelium-like epithelial cells, some of which contain karyokinetic figures. In the basal parts of the epithelial cells one sees nearly everywhere fine-grained fat (fig. 35) which mostly is

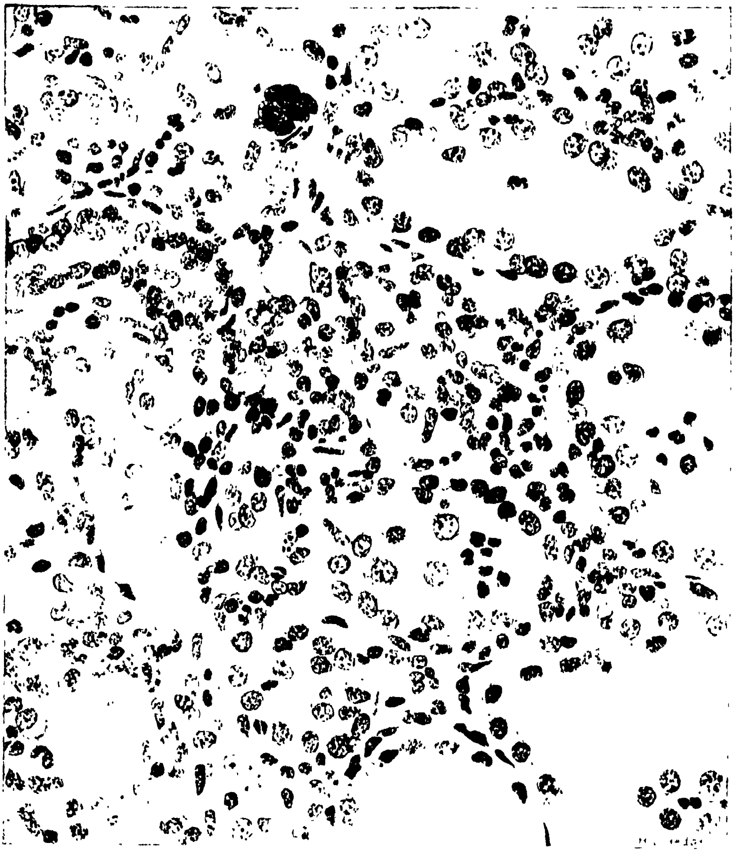
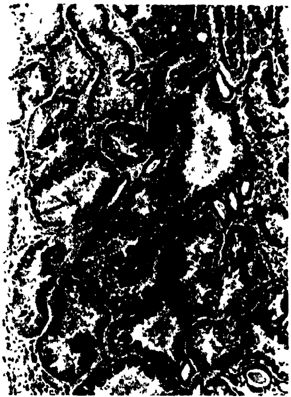


FIG. 35. Case 59. For diagnosis see figure 33.

Xanthoma-like lipoid cells in the interstitial tissue. Fatty degeneration of the convoluted tubules in the lumina of which one sees desquamated epithelial cells and polymorphonuclear leucocytes. Mueller-formalin fixation, Sudan III-hematoxylin. ×400.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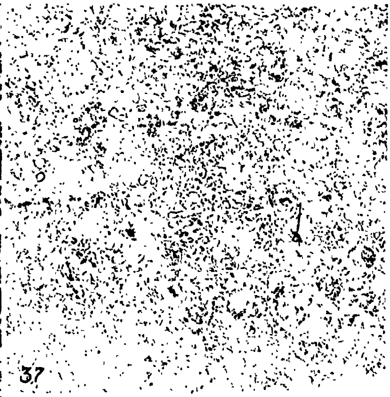
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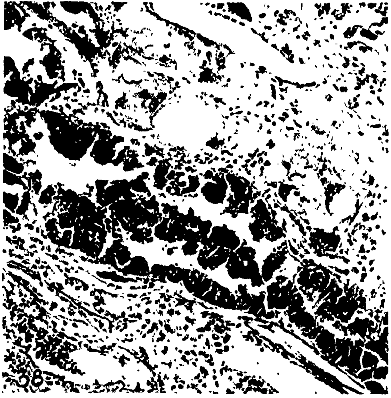
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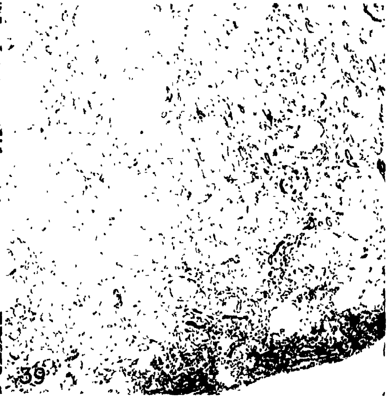
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FIG. 36. Case 59. For diagnosis see figure 33.

Regeneration in a gap of the epithelial layer (*a*). In the lumina of the tubules coagulated protein and some polymorphonuclear leucocytes (*b*). Mueller-formalin fixation, iron-hematoxylin-eosin. ×160.

FIG. 37. Case 63. Anatomical diagnosis: amyloid nephrosis, Stage II. Clinical diagnosis: non-hemorrhagic degenerative Bright's disease and tuberculosis.

Marked amyloidosis of the glomeruli and arterioli (*a*). Fatty and hyaline-droplet degeneration of the convoluted tubules. Mueller-formalin fixation, iron-hematoxylin-eosin. ×60.

FIG. 38. Case 63. For diagnosis see figure 37.

Extensive hyaline-droplet degeneration of some tubules. Mueller-formalin fixation, Gram. ×200.

FIG. 39. Case 66. Anatomical diagnosis: amyloid nephrosis, Stage III. Clinical diagnosis: non-hemorrhagic degenerative Bright's disease with renal failure.

Extensive diffuse amyloidosis of the glomeruli, arterioli, and arteries. Scar formation below the capsule with round-cellular infiltrations. Atrophy and degeneration of the tubules with numerous casts. Mueller-formalin fixation, iron-hematoxylin-eosin. ×60.

doubly refractive. Within the lumina of the tubules one finds coagulated protein, some desquamated epithelial cells, and in some places also polymorphonuclear leucocytes (figs. 35 and 36). The minor parts of the tubules contain fairly large numbers of casts which increase in number towards the medulla. The interstitial tissue of these parts of the cortex appears to be normal in most places.

In other parts of the cortex, the interstitial tissue is frequently enlarged and contains increased collagenous connective tissue. In places there are small round cell infiltrations, some of which are adjacent to hyalinized glomeruli. In other places, the interstitial tissue is swollen and penetrated by polymorphonuclear leucocytes which frequently enter the tubules. Everywhere in the interstitial tissue of these parts of the cortex one finds large, xanthoma-like cells, which are stained yellowish by Sudan (fig. 35), and which are doubly refractive. The tubules of the scarred parts are in all stages of atrophy and degeneration up to entire disappearance. Their epithelial cells are much more desquamated than those of other parts of the cortex. They frequently contain a large amount of fat which is very irregularly arranged. Occasionally one sees signs of regeneration. In some places one finds calcified casts.

Half of the glomeruli are well preserved (fig. 34), partly of normal size and *partly enlarged.* The enlarged glomeruli contain increased numbers of nuclei, but the increase is proportional to the size of the glomeruli. In a few places one finds small accumulations of endothelial cells in a single tuft. The tufts of the well preserved glomeruli are delicate and mostly well filled with blood. Occasionally, the capsule spaces are dilated and contain coagulated protein. There is little fat to be found in the covering cells of the glomeruli. Nowhere in these glomeruli does one see typical inflammatory lesions.

The other half of the glomeruli are in all stages of hyaline degeneration. In some otherwise well preserved glomeruli the capsules are concentrically thickened. Other glomeruli contain hyalinized tufts, especially at the entrances of the vessels; occasionally the tufts are adherent to the capsules. A few of these glomeruli show a slight proliferation of the parietal epithelial cells, but nowhere crescent formations. About one-third of the glomeruli are entirely hyalinized and are sharply separated from their surroundings.

The medulla is rich in collagenous connective tissue, which is swollen in places and contains some polymorphonuclear leucocytes and swollen round cells. In the tubules of the medulla numerous casts are seen, and in one place also some erythrocytes. The arterioli are well preserved. The larger arteries show a moderate degree of intimal hyperplasia. All amyloid reactions are negative.

Anatomical diagnosis. Lipoid nephrosis.[5]

Case 60. Hospital No. 5159. M. R., male, 30 years. *Degenerative Bright's disease.*

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[5] A more detailed description and discussion of this case see in MacKay and Johnston (1930), and Ehrich (1930).

Sixteen months before admission edema and albuminuria appeared and have remained since then.

On admission the urine sediment contained no erythrocytes but some hyaline and granular casts. The eyegrounds, blood pressure and heart size were normal. Wassermann reaction was negative. Plasma globulin is 2.62 per cent, albumin 2.17 per cent, blood urea nitrogen 19 mgm. per cent, blood creatinine 1.57 mgm. per cent, plasma cholesterol 417 mgm. per cent, basal metabolic rate −30.6 per cent.

During stay in hospital the urine sediment usually contained many hyaline and granular casts. The plasma albumin varied between 1.62 and 2.19 per cent. The other findings were unchanged, up to the time of discharge. Subsequent examinations, made 13 months and 16 months afterwards showed little change.

Case 61. Hospital No. 5505. D. B., male, 9 to 10 years. Height 134 cm. *Degenerative Bright's disease.*

Two months before admission the patient had a slight cold followed by swelling of the face and legs and by albuminuria.

On admission the urine sediment contained hyaline and granular casts and a few leucocytes and doubly refractive bodies. The eyegrounds, blood pressure and heart size were normal. Chronic tonsillitis was present but no sinusitis. Wassermann reaction was negative. Plasma globulin was 2.52 per cent, albumin 1.31 per cent, blood urea nitrogen 32 mgm. per cent, blood creatinine 1.23 mgm. per cent, total fat in plasma 1.32 per cent and the basal metabolic rate −13.5 per cent.

These findings did not change significantly during the period of observation except for the sediment, which, when the patient was last seen, 20 months after admission, contained no casts but still some leucocytes. At this time the patient was going to school and in good condition. He is still taking a salt-free diet. The septicemia 6 months after admission was caused by pneumococcus Type IV.

Case 62. Hospital No. 5913. M. H., female, 23 years. *Degenerative Bright's disease.* Complicated by pregnancy.

Seven months before admission albuminuria was accidentally found. One month later she became pregnant and during the next month edema appeared. An abortion was provoked, but the edema persisted.

On admission the urine sediment contained no erythrocytes, some leucocytes and hyaline casts, and many granular casts. The eyegrounds, blood pressure and heart size were normal. Wassermann reaction was negative. Plasma globulin 1.88 per cent, albumin 1.88 per cent, blood urea nitrogen 36 mgm. per cent, blood creatinine 1.51 mgm. per cent, plasma cholesterol 536 mgm. per cent, basal metabolic rate −16 per cent.

During observation the number of casts decreased considerably. The plasma albumin increased to 1.94 per cent. The patient is still taking a salt-free diet.

Case 63. Hospital No. 6510. J. B., male, 46 years. *Degenerative Bright's disease (amyloid) with tuberculosis* → exitus.

For 3 years before coming to hospital, the patient had had severe gastrointestinal symptoms. Six months before admission edema of the feet and legs had appeared and he was told that he had Bright's disease because of the finding of albumin in the urine. He had lost 20 pounds in weight during the 3 months previous to the onset of edema. At no time had there been gross hematuria.

On admission there were signs of advanced pulmonary tuberculosis. The blood pressure measured 120 mm. systolic and 80 mm. diastolic. The urine contained about 18 grams of protein per day, many hyaline casts, double refractive bodies, and many white blood and epithelial cells; no red blood cells were observed in the sediment. The blood urea N was 17.1 mgm. per cent, and the plasma NPN was 36 mgm. per cent. The albumin fraction of the plasma proteins was only 0.87 gram per 100 cc. The globulin was increased to 4.4 per cent. The blood hemoglobin was 16.5 volumes per cent O_2 capacity. A phenolsulfonephthalein test resulted in a 70 per cent return of the dye in 2 hours.

During the 3½ months that the patient was under observation, the pulmonary lesion increased. Evidence of degeneration in the kidneys continued: the protein excretion in the urine remained above 20 grams per day, and the excretion of white blood and epithelial cells and casts increased. Excretion of red blood cells did not occur. The plasma proteins remained low, as did the blood pressure. The phthalein excretion was reduced to 38 per cent in 2 hours. The blood urea nitrogen did not rise above 27 mgm. per cent. The development of intestinal tuberculosis lesions, during the second month that the patient was in the hospital, hastened death.

Autopsy. Autopsy No. 313/1928. We were permitted to do only an autopsy of the abdominal cavity. This was performed 13 hours after death.

Death by chronic pulmonary and ulcerous intestinal tuberculosis. Extended amyloidosis was present also in the kidneys, in liver, spleen, adrenals and intestine.

The right kidney weighed 210, the left 220 grams. Both kidneys were firm. Their capsules stripped easily. The surface of each was smooth and yellowish gray in color. The cortex was enlarged and similar in color to the surface. It was well marked off from the brown red medulla.

Microscopic examination. All glomeruli are more or less diseased. In each glomerulus all or part of the loops are infiltrated with amyloid in diffuse distribution (fig. 37). The amyloid gives a positive iodine sulfuric acid reaction as well as a positive methyl violet reaction. Frequently the glomeruli contain tufts well filled with blood. There is no fat within the glomeruli. Occasionally one finds increase in the number of nuclei in some tufts. The parietal epithelial cells are increased in number in some instances.

Nearly all arterioli are changed in to thick amyloid tubes (fig. 37), the lumina of which are open in most places. Occasionally they contain fat. The larger arteries contain only a little amyloid and show slight degree of intimal hyperplasia.

The tubules of the cortex are markedly changed. The *proximal tubules* are enlarged and lined with greatly swollen epithelial cells which in places close the lumina entirely. The epithelial cells have undergone degeneration, which is partly of the fatty, partly of the hyaline-droplet type (fig. 38). Frequently they contain pycnotic nuclei or have lost them entirely. In many instances the epithelial cells are desquamated. In such places there are signs of regeneration. In the basal parts of the epithelial cells one sees nearly everywhere fine or coarse grained fat, part of which is doubly refractive. Also the *distal parts* of the tubules contain in places much fat. In the lumina of the tubules one finds some coagulated protein, many swollen and desquamated epithelial cells, and in some places also polymorphonuclear leucocytes. Everywhere they contain numerous casts.

The interstitial tissue is well preserved. In a few places small scars are seen which may contain small round cellular infiltrations.

Anatomical diagnosis. Amyloid nephrosis (Stage II of Fahr).

Case 64. Hospital No. 5219. B. F., female, 24 years. *Degenerative Bright's disease* → exitus.

Ten months before admission edema gradually appeared, followed by loss of strength and appetite. Two months before admission the patient had pleurisy for 3 weeks.

On admission marked asthenia and some abnormal increase in skin pigmentation was found. The urine sediment contained some leucocytes and hyaline casts. The eyegrounds were normal except for pigmentation. The blood pressure was 98/76. The heart was a "drop-heart." A test meal showed achylia gastrica. Wassermann reaction negative. Plasma globulin was 2.08 per cent, albumin 1.77 per cent, blood urea nitrogen 25 mgm. per cent, blood creatinine 1.27 mgm. per cent and the basal metabolic rate 11.0 per cent.

During observation the urine sediment showed an increasing number of granular casts. Occasionally doubly refractive bodies were found, but never any erythrocyte casts. The blood pressure remained abnormally low until 5 months after admission; it then rose to about 125/80, but decreased again during the last 8 months of observation. Diarrhoea was often present, and anorexia and asthenia most of the time. Amenorrhoea was present during a period of 5 months. The plasma albumin gradually increased to about 2.4 per cent, but one week before death it was only 1.82 per cent. Five months after admission the blood urea nitrogen began to rise. One week before the death the blood urea nitrogen was 211 and the creatinine 9.1 mgm. per cent. Death occurred in asthenia after a period with diarrhoea and oliguria. The day before death the blood pressure was 66/38. No autopsy. Addison's disease was suspected.

Case 65. Hospital No. 5949. M. K., male, 20 years. *Degenerative Bright's disease → uremia →* exitus.

For several years he had a chronic infection of the nose. This practically ceased after an operation 5 months before admission. Eight months before admission edema and ascites appeared. Three months later albuminuria was found, but the blood pressure and blood urea nitrogen were normal.

On admission the urine sediment contained a few erythrocytes, some leucocytes, many granular and cellular casts, and occasional doubly refractive bodies. The eyegrounds, blood pressure, and heart size were normal. Wassermann reaction negative. Plasma globulin was 2.47 per cent, albumin 1.30 per cent, blood urea nitrogen 35 mgm. per cent, blood creatinine 1.46 mgm. per cent, plasma cholesterol 877 mgm. per cent, and basal metabolic rate −28.2 per cent.

During observation the plasma albumin decreased further to a minimum of 1.17 per cent and then rose gradually to 1.80 per cent. The plasma cholesterol decreased and was 226 mgm. per cent 9 months after admission. The urine sediment and blood pressure had not changed significantly. The blood urea nitrogen increased to 99 mgm. per cent, the blood urea clearance fell to values characteristic of the terminal stage and death followed in uremia. No autopsy.

Case 66. Hospital No. 6834. F. A., male, 42 years. *Degenerative Bright's disease (amyloidosis) → uremia →* exitus.

The patient passed an insurance examination 3 years before admission and was told he had abnormally low blood pressure at that time. Onset 17 months before admission with weakness. Edema appeared 5 months later. There had been no gross hematuria noted. From 2 months previous to admission there had been severe abdominal cramps, also intermittent diarrhea.

On admission to hospital the patient was in uremia. The blood urea nitrogen was 86.1 mgm. per cent. Non-protein nitrogen 115 mgm. per cent. The phenolsulphonephthalein test resulted in less than 1 per cent return in 2 hours. The blood pressure was 110/55. The plasma albumin was somewhat reduced to 3.1 per cent, the globulin remaining normal at 2.7 per cent. A fresh specimen of urine revealed by ordinary microscopic examination no red blood cells. However, a concentrated 12-hour specimen showed 1,600,000 red blood cells (normal limit = 500,000), 27,000,000 white blood cells and epithelial cells, and 600,000 casts, 10 per cent of which were of Addis' renal failure type, 5 per cent hyaline, 5 per cent epithelial, and 80 per cent granular in character. The protein excretion in the urine was 3.5 grams daily. While under observation, the patient's condition became rapidly worse, and he died of uremia on the tenth day. The blood pressure remained low to the end; the systolic measured between 90 and 110 mm. and the diastolic between 55 and 78 mm.

Autopsy. Autopsy No. 317/1929. Autopsy commenced 8 hours after death.

Death in uremia. Uremic hemorrhages in the intestine. Extended amyloidosis, in the kidneys, liver, lung, adrenals, pancreas, spleen and intestine. Fresh serofibrinous pericarditis. Old healed endocarditis of the mitral and aortic valves. Moderate atheromatosis of the aortic cusp of the mitral valve, of the coronary arteries and of the aorta. An etiologic cause for the amyloidosis could not be found.

The right kidney weighed 160, the left 170 grams. The capsule of each stripped easily. Both kidneys were remarkably flabby. The cortex was yellowish gray in color and finely granulated. Hemorrhages were not seen. The cortex was of about normal breadth, yellowish gray in color and faded. It was well marked off from the medulla.

Microscopic examination. The glomeruli are of about normal size.

Frequently, they fill out the capsules entirely. *Their tufts are greatly infiltrated with amyloid in diffuse distribution* (figs. 39 and 40). The amyloid no longer gives iodine sulfuric acid reaction, but does give a positive methyl violet reaction in many places. Only a few glomeruli still contain blood. Most of the glomeruli do not show any erythrocytes. Many tufts are poor in nuclei. Many glomeruli contain much fat in their tufts (fig. 41). The parietal epithelial cells are occasionally increased in number. Single glomeruli are hyalinized and stained red by Van Gieson.

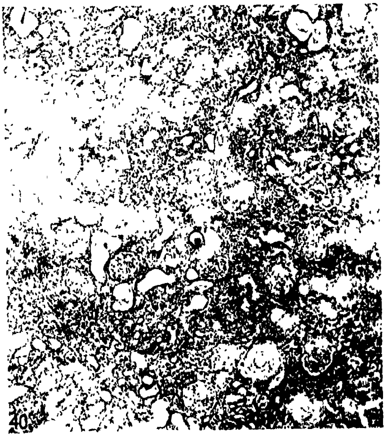

FIG. 40. Case 66. For diagnosis see figure 39.

Extensive diffuse amyloidosis of the arteries, arterioli, and glomeruli. Distinct scar formation in the interstitial tissue. Atrophy and degeneration of the tubules. In the tubules many casts. Mueller-formalin fixation, elastica-Van Gieson. ×60.

Nearly all arterioli are changed into thick amyloid tubes (figs. 39 and 40), the lumina of which are narrowed or closed entirely. Frequently they contain fat. Also the larger arteries are much infiltrated with amyloid. In most places all layers of the walls are affected. However, the largest amount of amyloid is found in the adventitial layer. Occasionally, the media is fairly well preserved. The intima of the larger arteries is hyperplastic in some instances and contains fat.

Also in the interstitial tissue of the kidneys, especially of the medulla, diffuse amyloid is found.

The tubules are in the stage of advanced atrophy up to entire disappearance. Frequently, they are dilated and their epithelial cells flattened so that it is often difficult to differentiate ascending and descending tubules. Occasionally, the epithelial cells contain fat which is very irregularly arranged. Vacuolic degeneration is distinct in some places, but as a slight hyaline-droplet degeneration, not very conspicuous. The epithelial cells are frequently desquamated. Occasionally one sees signs of regeneration. In the lumina of the tubules one finds coagulated protein. Of the epithelial cells some are desquamated and some have undergone fatty degeneration. In a few places there are polymorphonuclear leucocytes. Everywhere the tubules contain large numbers of casts.

The interstitial tissue of the kidneys is frequently enlarged and contains increased collagenous connective tissue. In such scars one sees round cell infiltrations (fig. 39). In a few interstitial places one finds some polymorphonuclear leucocytes entering the tubules. The capillaries are in part well filled with blood.

Anatomical diagnosis. Amyloid nephrosis (Stage III of Fahr).

SUMMARY

Clinical, chemical and functional observations, continued for periods varying from a few weeks to several years, are reported on 67 patients with Bright's disease, hemorrhagic, sclerotic, and degenerative. Gross and microscopic anatomical findings are described for 17 of these cases.

Our observations support the view of Volhard and Fahr and of Addis that the three types of Bright's disease are essentially different in their genesis and pathological nature: the hemorrhagic or glomerular, marked primarily by glomerular inflammation, with hematuria and usually diminished renal function (even in the acute stage); the sclerotic disease, marked primarily by pathological changes in the small arteries of the kidneys (and usually other organs), with hypertension as the first sign and diminished renal function only as a terminal phenomenon; the degenerative disease or diseases, called nephrosis, marked primarily by degenerative changes in the kidneys, without hypertension and with hematuria. Contrary to the apparently general belief, except that of Addis, we have observed that gradual decrease of urea excreting ability frequently develops during the course of nephrosis, and that the disease may end in uremia, the glomeruli being then involved in the degenerative changes.

Our data also support the belief of Volhard and Fahr and of Addis that it is possible from observations during the course of the disease to deduce the general nature of the pathological changes occurring

FIG. 41. Case 66. For diagnosis see figure 39.

The tufts of the glomerulus are infiltrated by amyloid and fat. Some of the loops contain dark-blue stained grains (calcium?). The afferent arteriole is entirely changed to amyloid matter. Mueller-formalin fixation, Sudan III-hematoxylin. ×200.

in the kidneys. In the cases clinically diagnosed as hemorrhagic nephritis and examined post-mortem, the changes characteristic of glomerular inflammation were outstanding, except in some diagnosed as hemorrhagic plus arteriosclerotic, in which it was difficult to tell histologically whether the glomerular or arterial changes were primary. In the cases diagnosed as arteriosclerotic and as degenerative Bright's disease (nephrosis) the post-mortem findings were confirmatory. The clinical differentiation between lipoid and amyloid nephrosis was not attempted.

The diagnoses have been made chiefly on the basis of observations of the urea excreting power of the kidneys, measured by the blood urea clearance (cubic centimeters of blood cleared of urea by 1 minute's excretion, Møller, McIntosh, and Van Slyke (1928)), of the hematuria, blood pressure, plasma protein content, proteinuria, and edema. Blood hemoglobin content was also followed, and although not of decisive diagnostic value was found to be of interest as an indication of the toxic damage suffered by the organism, and to have prognostic significance. The above observations are presented in charted form for each patient.

In acute hemorrhagic nephritis the prognosis was found to be independent of the severity of the disturbances during the first weeks, with the single exception of the plasma albumin content. The majority of cases in which this fell to a low level became chronic. Intensity of hematuria, proteinuria, and degree of hypertension had no apparent relation to the probability of recovery. Fall of the renal function to as low as 10 per cent of normal, measured by the blood urea clearance, was found not inconsistent with apparently complete recovery. The majority of cases showed during the first two months a decided fall in renal function measured by the blood urea clearance. In all these cases which recovered or improved, however, the blood urea clearance began to rise within 4 months after the acute hemorrhagic onset. The occurrence or non-occurrence of this rise in renal function constituted the most definite single prognostic sign.

After the initial acute stage of hemorrhagic nephritis a period was observed in some cases during which hematuria and hypertension quite disappeared, while proteinuria, plasma protein deficit, and edema still persisted. During this stage, these cases, except for their acute hemorrhagic history, were indistinguishable from nephrosis. Subsequently in a few months, however, they either completed recovery or improvement to the symptom-free latent stage, either of which was a relatively rare occurrence in our nephrosis cases, or else they developed into chronic hemorrhagic nephritis.

The tendency to non-cardiac edema was found to parallel approximately the fall in albumin content of the blood plasma, except during the first weeks of acute hemorrhagic nephritis. Edema during this period was repeatedly observed even when the plasma proteins remained normal. The edema was, however, moderate and temporary unless plasma albumin deficit developed. In all stages of nephrosis plasma albumin deficit and tendency to edema occurred together. One case of amyloid nephrosis, the diagnosis of which was checked by autopsy, was exceptional in that edema had been present to only a slight extent: in this case the plasma albumin showed only a relatively moderate fall below normal. In arteriosclerotic renal disease plasma proteins were never markedly reduced, and only cardiac edema was observed.

Of the different features of the disease that were followed, the blood urea clearance proved to be the most closely related to the onset of final renal failure. The renal function, measured by the clearance, could apparently remain indefinitely at 10 per cent of normal without uremia; but when it fell to below 5 per cent uremia occurred and was usually fatal. Exceptions to the immediately fatal outcome were found in acute cases, which can recover if the functional depression does not last too long, and occasionally a terminal case, in which the functional fall is partly due to factors, such as desiccation, other than destruction of renal tissue. In such a case treatment, particularly saline and glucose injections, may both improve general condition and somewhat increase the blood urea clearance, although the added lease of life appears to be at most a few months.

The above summary indicates only incompletely the nature of the observations reported. The variations within each of the 3 types of Bright's disease studied are great, and a study in detail of the case examples presented is necessary for utilization of the material concerning the incidence, intensity, time relationships, and diagnostic and prognostic significances of the functional and chemical disturbances that have been studied.

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A COMPARISON OF THE UREA NITROGEN CONTENT OF CUTANEOUS AND VENOUS BLOOD BY MICRO GASOMETRIC ANALYSIS

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Svensgaard (1) using the micro urea method of Rehberg (2) compared the urea concentrations in blood from the arm vein with that of cutaneous blood from the lobe of the ear. In 29 of 32 analyses of the blood of normal fasting subjects she found the urea of the capillary blood higher than that of the venous blood. The average difference was 10 per cent of the venous urea content, but individual differences were as high as 24 per cent. Such results, if confirmed, would indicate that the use of cutaneous blood from the ear for analysis could introduce errors up to 24 per cent in tests of renal function, such as the blood urea clearance used in this clinic (3, 4, 5), which depend upon blood urea determinations.

It was therefore thought advisable to make a series of determinations of the urea content of samples of cutaneous and venous blood taken simultaneously and both analyzed by the Van Slyke micro gasometric method (6) in order to ascertain whether the variations found by Svensgaard could be confirmed. A report of the analyses is given in this paper.

METHODS

Into each of two small tubes (10 mm. inner diameter and 50 mm. in length) a little powdered heparin was introduced. The tubes were then rotated horizontally until the heparin was evenly distributed, and were then inverted so that only the finely powdered heparin which clung to the walls of the tube remained. After preliminary rubbing to produce hyperemia, the lobe of the ear was punctured deeply enough to insure a free flow with only slight pressure, and sufficient blood was collected in one tube. From time to time during the collection the puncture wound was wiped clean so that only fresh blood would be obtained. Vena puncture was done immediately afterward, either with or without stasis. The samples were taken immediately to the laboratory and 0.2 cc. portions measured

TABLE 1

Comparison of the urea nitrogen content of cutaneous and venous blood

Subject	Venous blood urea nitrogen			Cutaneous blood urea nitrogen			Absolute difference venous minus cutaneous	Difference as per cent of venous	Remarks
	Individual analyses	Mean	Maximum deviation	Individual analyses	Mean	Maximum deviation			
	mgm. per 100 cc	*mgm. per 100 cc.*	*per cent*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*per cent*	*mgm. per cent*		
W. N.	9.2 9.5 *	9.4	2.1	9.5 9.6 9.7	9.6	1.0	−0.2	2.1	Normal
A. H.	9.6 10.0	9.8	2.0	10.1 9.8	10.0	2.0	−0.2	2.0	Normal
R. H.	10.2 10.0 *	10.1	1.0	10.0 10.1 10.2	10.1	1.0	−0	0	Normal
W. K.	11.5 10.5 10.7	10.9	6.0	11.3 12.0 11.9	11.7	3.4	−0.8	7.3	Normal
M. L.	13.8 13.8 13.8	13.8	0	14.1 13.6 14.1	13.9	2.2	−0.1	0.7	Normal
A. S. A.	14.8 15.1 14.9	14.9	1.3	15.0 14.7 14.6	14.8	1.4	+0.1	0.7	Normal
A. S. A.	17.1 17.4 17.7	17.4	1.7	16.8 17.6 17.3	17.2	2.3	+0.2	1.2	Normal
A. C.	9.6 9.4 9.6	9.5	1.1	9.3 9.7 9.7	9.6	3.1	−0.1	1.1	Nephritic, during diuresis
J. D.	40.8 41.3 40.8	41.0	0.8	40.4 39.8 38.9	39.7	2.0	+1.3	3.2	Nephritic, urea therapy

Average of all venous bloods 42.4 mgm. per cent. Average of all cutaneous bloods 42.4 mgm. per cent.

* Discarded because of gross error.

TABLE 1—*Concluded*

Subject	Venous blood urea nitrogen			Cutaneous blood urea nitrogen			Absolute difference venous minus cutaneous	Difference as per cent of venous	Remarks
	Individual analyses	Mean	Maximum deviation	Individual analyses	Mean	Maximum deviation			
	mgm. per 100 cc.	*mgm. per 100 cc.*	*per cent*	*mgm. per 100 cc.*	*mgm. per 100 cc.*	*per cent*	*mgm. per cent*		
J. E.	63.6	63.9	0.6	64.0	63.8	0.3	+0.1	0.2	Nephritic, urea therapy
	64.3			63.7					
	63.7			63.8					
M. G.	101.1	101.8	0.7	103.9	103.8	0.2	−2.0	2.0	Nephritic. Uremia
	102.1			103.6					
	102.2			*					
D. McK.	117.9	118.3	0.3	116.6	117.2	0.5	+1.1	0.9	Nephritic. Uremia
	118.7			117.3					
	*			117.8					
J. O'M.	129.9	130.1	0.4	130.1	130.1	0.7	0	0	Nephritic. Uremia
	129.9			131.0					
	130.6			129.2					

into tubes containing 1 cc. of 0.02 N lactic acid as described by Van Slyke. As analyses were made in triplicate, three accurately calibrated pipettes were used. After taking the three samples of blood from one source they were washed and thoroughly dried before using again. After addition to lactic acid, the samples stood at room temperature. As each analysis takes 15 minutes, and as at least two blank determinations were made with each experiment, the last sample stood approximately 2 hours before analysis, but it seemed to make no difference.

The method of collecting blood from the ear into a tube rather than by drawing directly into a pipette was chosen because it is easier, and measurement of the 0.2 cc. samples can be made more comfortably and hence with greater accuracy in the laboratory. There is no trouble from clotting.

Bleedings were done between one and two hours after lunch, except in three experiments, which were done about the same length of time after breakfast. No attempt was made to control diet.

RESULTS

Inspection of table 1 shows that the gasometric method, using 0.2 cc. of whole blood, gives results of sufficient accuracy to demonstrate any essential difference in the concentration of urea nitrogen between cutaneous and venous blood. Occasionally, through faulty technic, one gets a reading that is obviously incorrect and has to be discarded. This happened but four times in this series of 72 analyses. In the case of subject W. K., it is likely that one of the stopcocks was leaking slightly, which led to the introduction of an error much larger than usual. For this reason the experiment should probably be discarded but it is included here for the sake of completeness. No experiments have been omitted from the table.

Excluding the observation in question, five of the remaining twelve sets of analyses showed a higher concentration in cutaneous blood (average 1.6 per cent), five showed higher concentration in venous blood (average 1.2 per cent), and two showed no difference. The average concentration in the twelve samples of both venous and cutaneous blood is 45.0 mgm. per cent urea nitrogen. The maximum difference in any one experiment was only 3.2 per cent.

DISCUSSION

Marshall and Davis (7) in a study of the distribution of urea in the body concluded that "the urea content of all organs and tissues is approximately uniform, and approximately equal to that of blood, both in normal conditions, and when there is an abnormally large amount of urea present." Furthermore "when urea in solution is injected intravenously, it diffuses to all parts of the body almost instantly, the diffusion being complete in a few minutes." Gad-Andresen (8) came to essentially the same conclusions with regard to equilibrium between tissues and blood, and in addition found that the distribution coefficient between body secretions and blood plasma was unity, with the exception of sweat and tears, in which the concentration of urea was higher than in the plasma. He found, however, that plasma contained more urea than the erythrocytes, the distribution coefficient being between 0.72 and 0.82. Both Marshall and Davis and Gad-Andresen found that fat is an exception to the general rule in that it is relatively poor in urea. The experiments of Bollman, Mann, and Magath (9) seem definitely to have established that urea formation is a function of the liver only. If equilibrium exists between blood and tissues, one would expect to find no difference in the concentration of urea between venous and cutaneous blood.

So far, no explanations have been found to account for the apparent differences noted by Svensgaard. The fact that the mean error of her tests was ±1 mgm. urea (0.47 mgm. urea N) might have contributed some, but the average difference noted was much greater than this. Returning again to her observations on normal subjects, the concentration of urea in capillary blood varied between 4 and 22 per cent higher than in venous blood. After administration of urea, or of protein the differences were from 1 to 7 per cent. In seven cases of icterus the variation was from 2 to 23 per cent, in five cases of nephritis from 1 to 13 per cent, and in 8 of 9 diabetics from 2.2 to 24 per cent.

SUMMARY AND CONCLUSIONS

1. The concentration of urea nitrogen in the blood from ear puncture has been compared with that from the vein in 13 experiments on 6 normal and 6 nephritic subjects, without regard to food intake.
2. No difference between venous and cutaneous blood exceeding the limit of experimental error was found to exist.

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STUDIES ON A-AVITAMINOSIS IN CHICKENS

I. LESIONS OF THE RESPIRATORY TRACT AND THEIR RELATION TO SOME INFECTIOUS DISEASES

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PLATES 21 TO 23

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The diseased condition of chickens resulting from a lack of vitamin A in the diet is probably the most important of the nutritional diseases caused by A-avitaminosis in domestic animals. As pointed out by Beach (1) and Seifried and Schaaf (2), it causes serious losses on poultry farms. The clinical and anatomical picture closely resembles that found in some of the most important infectious diseases of fowls, chicken pox, coryza contagiosa, and infectious bronchitis. Golblatt and Benischek (3), Tyson and Smith (4), and Wolbach and Howe (5) have described the histological changes following a lack of vitamin A in the diet in guinea pigs, rats, and in one human individual. The characteristic changes are the substitution of stratified keratinizing epithelium for normal epithelium in various parts of the respiratory tract, alimentary tract, eyes, paraocular glands and the genitourinary tract. No studies have been made of the histological changes in A-avitaminosis in fowls. It is the purpose of this paper to report such studies and to compare the changes with those found in certain infectious diseases.

Material

The material used consisted of 16 cases of A-avitaminosis, of which 12 were produced experimentally and 4 were spontaneous. The diet employed for the experimental production of the disease was identical with that used by Beach and

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TABLE 1

Showing Source and Kind of Material Used

Number of chicken		Food received	Age at beginning of experiment	First symptoms after	Dead after beginning of experiment	Gross lesions											Histological findings
						Eyes	Nasal fossa, cleft palate	Sinuses	Larynx	Trachea, bronchi	Cavity of the mouth, (excretory ducts of the glands)	Esophagus, crop	Proventriculus	Urates (kidney)	Urates (visceral)	Other lesions	
				days	*days*												
1	Experimental cases	Food mixture I alone	About 3 months		30 (Killed)	+	+++	+	+	+	–	–	–	–	–	–	Positive
2		"	"	40	70	++	+++	++	++	+++	++	+++	+	++	–	Oviductitis	"
3		"	"	45	76	–	++	++	++	+++	++	+++	–	+	–	–	"
4		"	"	48	79	–	++	++	+	++	+	++	–	++	+	–	"
5		"	"	44	85	++	+++	+++	+	++	++	+++	+	–	–	–	"
6		"	"	52	87	–	++	++	++	++	+	+	–	+	–	–	"
7		"	"	51	82	++	++	++	+	+++	+++	+++	–	–	–	–	"
8		"	"	58	80	++	++	+	–	++	+	+	–	–	–	–	"
9		"	"	54	75	±	+++	+++	++	+++	++	++	+	+	+	Oviduc-	"
10		"	"	59	77	+	+++	++	++	++	+	–	–	–	–		
11		"	About 6 months	–	56 (Killed)	–	±	±	–	–	–	–	–	–	–		
12		"	"	–	81 (Died suddenly)	–	+	+	–	–	–	–	–	–	–	–	" *

13	Spontaneous cases	Lack of vitamin A	?	?	–	+	+++	+++	++	+++	–	–	–	–	–	Oviductitis	Positive
14		"	?	?	–	–	++	++	+	++	+	–	–	–	–	–	"
15		"	?	?	–	++	+	+	–	–	++	++	+	+	+	–	"
16		"	?	?	–	–	+	+	–	–	++	++	–	–	–	–	"
17–24	Controls	Food mixture I + cod liver oil + greens	6 about 3, 2 " 6 months old	–	–	–	–	–	–	–	–	–	–	–	–	–	Negative

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* Main lesions in submaxillary and lingual glands.

by us in our earlier experiments. It consisted of 50 parts wheat bran, 50 parts wheat shorts, 50 parts ground barley, 10 parts meat scrap, 10 parts fish scrap, and 20 parts soybean meal. We used chickens about 3 months old, for according to our earlier experiments and in common with other investigators, we h[illegible]e observed that a much shorter period is required to produce the characte[illegible] symptoms followed by death in young, partially grown animals, than [illegible]. This is brought out in the present experiments in which Chickens 1[illegible] Table I) that were about 3 months older than the other animals, [illegible] symptoms much later and to a minor degree than did the younger chicks. To insure the presence of vitamin D, all animals were exposed daily to sunlight or to ultraviolet light. The six controls (Nos. 17–24) received the same food mixture and in addition plenty of greens (alfalfa, cabbage) and 3 cc. of cod liver oil daily given with a dropper. All of the controls remained well, failing to show any gross pathological or histological lesions. No symptoms of rickets or of a deficienc[illegible] f vitamin B were observed at any time during the experiment. Table I gives all details of the experiment together with the gross pathological findings.

Methods

Most of the tissues were fixed in 10 per cent formalin immediately after autopsy, but in a few cases Zenker's fluid was used. The tissues from the nasal passages and the sinuses were decalcified, some in 5 per cent nitric acid and some in 5 per cent formol-nitric acid. All tissues were imbedded in paraffin, and for special purposes frozen sections were made. For routine stains hematoxylin and eosin, van Gieson's stain, and Mallory's methylene blue phloxine, as modified by Davidoff (6) and McGregor (7), were used. In addition the following stains were used for special purposes: methylene blue, Gram's and Giemsa's stain,—the best results being obtained with the last, with tissues fixed in formalin as well as in Zenker's fluid, by the modification given by Wolbach,—Weigert's elastic tissue stain, Weigert's fibrin stain, fat stain with Sudan III and Scharlach R, oxydase reaction, the stains with methylgreen-pyronin and fuchsin-light green, with mucicarmine (Mayer), thionin, toluidin, cresylecht-violette, and Pasini's method with waterblue-orcein, which gave distinct results in staining the keratohyalin globules, and finally stains with ferric sulfur, calcium permanganate, Congo red (keratinization), and Hammerschmidt's stain for Guarnieri's inclusion bodies.

Gross Pathology

Nasal Passages and Sinuses.—Sagittal and gross sections through the nasal cavities and the communicating sinuses show all stages of a disturbance of the lining of the nostrils and cleft palate.

Early in the disease the turbinate bones (concha superior and inferior) are filled with seromucoid, water-clear masses, which by the application of slight pressure can be forced out of the nostrils and cleft palate. Owing to the consistency of the exudate and the complicated structure of the nasal fossae, the vestibule becomes plugged and the exudate overflows into the paranasal sinuses, often early in the disease. On the other hand, the exudate may be forced through the cleft palate, filling up the region around the vomer with seromucoid masses. Later this exudate becomes transformed into white or slightly yellow caseous masses which are found between the turbinate bones, in the nasolacrimal duct, nasal vestibule, and sinuses, causing more or less swelling of the face (Figs. 1 and 2). This involvement of the nasal passages and the sinuses may be uni- or bilateral. If on one side, the turbinate bones and the sinuses may become so enlarged that they extend over the median line and compress the opposite nasal fossa. The characteristic eye lesions in this disease, which will be described in another paper with M. Westhues, are complicated by the blocking of the nasolacrimal duct. When the sinuses are completely filled, the bulbus of the eye, which cannot move in a ventral direction, is pressed against the os frontale and sometimes forced out in a lateral direction. The protrusion of the palate into the cavity of the mouth, often observed in virus-diphtheria, is not usually seen in A-avitaminosis. After the inflammatory products have been removed, the mucous membranes throughout the nasal passages and sinuses show a peculiar, thin, rough and dry membrane, which may also be found in the mouth and throat. Unattached masses of white caseous material are often present in the cleft palate and in the surrounding mucous membrane of the roof of the mouth.

Larynx and Trachea.—Almost regularly in A-avitaminosis of chickens marked lesions occur also in the larynx, trachea and bronchi. As shown in the table, they are found in the early as well as in the later stages of the disease.

Chickens 11 and 12 on a diet lacking vitamin A showed no gross lesions, but microscopically early changes were found in the glands of the mouth, esophagus and trachea. Those in the trachea were apparently the most marked and were probably the oldest. The lesions in the trachea have as yet received no attention, but we feel that they were of special importance as it is so difficult to distinguish them from those commonly found in roup, diphtheria, and infectious tracheitis. In addition to the lesions near the entrance of the pharynx, consisting of pustule-like patches which are due to collections of white caseous material in the mucous glands, glandula cricoarytenoidea, the pathologico-anatomical picture is characterized by a peculiar laryngitis, tracheitis, and bronchitis. The mucous membrane of the anterior end of the larynx on its ventral side and in the pointed angle which is formed by the cartilages of the larynx, often shows caseous crumbly white masses. Similar lesions are frequently found throughout the entire length of the trachea and often in the bronchi. In very early stages, however, they are much less marked and may be difficult to see. The mucous membrane is then covered with a fine film or haze; it is dry, dull, and slightly uneven on the surface, whereas the normal membrane is moist. In other cases small nodule-like particles may be visible in or beneath the mucous membrane, especially in the upper part of the trachea. In later stages the gross lesions are much more striking and may easily be seen with the naked eye. More or less thin membranes cover the mucous membrane throughout the entire length of the trachea and the bronchi. In most cases these membranes are pulled off the underlying mucosa, thus forming a thin-walled continuous tube within the trachea and bronchi (Fig. 3), a condition that might be mistaken for infectious tracheitis. The smaller bronchi very often become completely plugged with these membranes causing bronchiectasis. In some cases the larynx shows the most marked changes while in others the trachea is more involved.

Histopathology

Larynx, Trachea, and Bronchi.—The changes in these structures are the more easily understood as the normal anatomy is less complicated than in the nasal fossae and adjoining sinuses.

The first lesion is in the columnar ciliated epithelium and is characterized by an atrophy of the cytoplasm and a loss of the cilia. Along with this protoplasmic change, the nuclei often present more or less marked karyorrhexis. The atrophying and degenerating ciliated cells hang like tufts on the basement membrane and later are pushed off and may form a pseudomembrane.

While this process is going on, there appear as islands beneath the original epithelium new cylindrical or polygonal cells either singly or in layers. These are especially marked in the trachea where they may appear as focal syncytial masses (Fig. 4). The cells evidently have their origin in the original columnar epithelium. They differ from the original cells in that their nuclei stain more deeply with basic dyes, and the presence of numerous mitoses indicates that they are dividing rapidly. Mitotic figures are especially numerous in the deeper layers of this newly formed epithelium. As these new cells become more numerous and form layers beneath or in place of the original epithelium, the cells nearer the surface are more and more flattened, the nuclei become larger and contain less chromatin. Later the superficial cells are quite flat, the cytoplasm is homogeneous and the nuclei are lost (Figs. 5 and 6). In this stage the cell boundaries become less clearly defined and at the surface the cells are desquamated as hard, dry, flattened scales. Pasini's waterblue-orcein method shows keratohyalin granules scattered irregularly in the cytoplasm of these cells. In the outer layers these granules sometimes fuse and form hyalin masses of eleidin. The process resembles very closely the incomplete keratinization of the mucous membranes, especially that of the esophagus and certain parts of the tongue of chickens. The columnar ciliated epithelium lining the trachea and bronchi and the epithelium of the submucous glands thus become transformed into squamous stratified keratinizing epithelium with typical intercellular bridges. Owing to the fact that the keratinized epithelium extends as a continuous layer into the glands of the submucosa, the picture strikingly resembles that of the mucous membrane of the cavity of the mouth, and to a less extent of the normal skin. This is brought out in Fig. 6. Eosinophilic leucocytes which are normally found in the keratinized epithelium of the tongue and esophagus appear either scattered or in small foci. Other cells which may be associated with secondary bacterial infections also appear.

Along with the formation of this keratinized epithelium the original columnar cells are thrown off either singly or in layers. Some of these cells may appear practically normal, but the majority lose their cilia, the protoplasm takes the routine stains less intensely, and the cells later become distended so that they have the appearance of a ball or a balloon (Fig. 7). The chromatin in the nuclei of these cells becomes irregular and fragmented, and later only a few chromatin knots remain scattered irregularly in the balloon-shaped cytoplasm. Finally the cells appear as more or less homogeneous shadows, the whole process being that described by Unna as "balloon degeneration." This balloon degeneration involves not only the original epithelium but also the squamous keratinizing cells. Occasionally these degenerating cells may fuse and form what may be called "balloon giant cells." The cell boundaries may still be visible in these unusually large giant cells.

In the deeper layers of the new epithelium a reticular degeneration of cells may be found. The cells are enlarged, the cytoplasm is occupied by round vacuoles, sometimes to such an extent that it is only visible as a reticular network, the nuclei show numerous chromatin clumps, and nuclear material may be extruded into the cytoplasm. This type of degeneration is not as common as the balloon type.

The degenerating cells become separated and collect in the lumen of the trachea and bronchi in the form of a membrane in which numerous bacterial colonies appear. These bacteria may invade the cells and in cases become so numerous that the cytoplasm is filled with them and the nuclei sometimes destroyed (Fig. 7).

Stains with Sudan III and Scharlach R of the trachea at various stages of the degeneration show practically no fat. In a few cases a small amount of fat was present but this type of degeneration is far from common.

As the keratinization of the epithelial cells of the glands in the trachea progresses, the presence of mucus, as shown by special stain, becomes progressively less. In the early stages one may find keratinized epithelium in one portion of the gland and secreting mucus cells in another. Later, as the keratinized epithelium replaces the true glandular epithelium, mucin is found, if at all, in very small granules in the center of what remains of the original epithelium. When the glands become completely filled with keratinized squamous epithelium mucin is absent.

Associated with the loss of cilia and the decreased or absent secretion of mucin one would expect an invasion of the mucosa with bacteria. Organisms are found not only on the surface of the epithelium and between the epithelial cells but also in the glands of the tunica propria, in the tunica propria itself, and even in the submucosa. The degenerating cells of the epithelium soon become invaded by bacteria and leucocytes may make their appearance. As the disease progresses bacteria and signs of inflammation become less marked. However, signs of bacterial infection are not always present, and in the fowl we feel that the keratinization of the epithelium is not related to the bacterial infection. In the rat, Tyson and Smith (5) came to the opposite conclusion.

In nearly all cases, a more or less marked cell infiltration in the tunica propria, partly around the numerous vessels, partly in a more diffuse distribution, is found (Figs. 5 and 6). According to the results of control investigations, the occurrence of lymphoid tissue in the tunica propria of the trachea is a normal finding, but in cases of A-avitaminosis its collection is so massive that there is no doubt of its pathological significance. The greater part of these infiltration cells are lymphocytes, to which are added typical histiocytes and a few plasma cells. In most cases some eosinophile leucocytes, red blood corpuscles and occasionally neutrophile leucocytes have also been found. In some early stages with extreme degeneration and proliferation of the original epithelium and a distinct edematous condition of the underlying propria, the occurrence of peculiar large cells, a type of histogenous mast cells, has been observed, especially in the upper layers. They are elongated or oval with a longish-oval nucleus which is rich in chromatin and sometimes shows a typical wheel form. Using Giemsa's stain, very small blue or blue-violet granules can be found in their cytoplasm. In general one has the impression that the upper layers of the tunica propria, situated directly beneath the epithelium of the surface, and the neighborhood of the glands are occupied more densely with these infiltrating cells than its deeper parts. Sometimes the reverse is true; in fact, the degree of the infiltration varies greatly in different cases. Mitoses are very seldom recognizable in these cells. Moreover, lesions may also be found in the endothelial cells of the infiltrated vessels, the lumina of which are frequently filled with lymphocytes. There is more or less swelling of these cells and a change in the chromatin structure of their nuclei. In some preparations the elastic fibers seem to be thickened and more separated. Usually these lesions are associated with edema. In the remaining parts of the trachea no particular changes could be discovered.

The same complex of lesions described in the trachea is also present in the bronchi and bronchioli. They are sometimes filled with desquamated keratinized epithelium, so that bronchiectasis is the natural sequence.

Nasal Cavities and Communicating Sinuses.—The lesions of the nasal passages and their sinuses have been studied on cross sections cut perpendicular to the roof of the mouth at several points between the nostrils and the posterior limit of the nasal cavity or bulb of the eye. The evolution, course, and final stage of the process are exactly the same as previously described in the trachea (Figs. 8, 9, and 10). It may be pointed out that in general all parts of the nasal cavities are involved and that only some chronological differences in the involvement of the several regions can be observed. In the gland-free part of the nasal vestibule, regio vestibularis, lesions have been determined in all cases, not so much in the form of a real keratinization but rather of an increased proliferation of the superficial epithelial cells.

Typical and distinct lesions, as described in the trachea, are found in the respiratory mucous membrane of the roof of the nasal vestibule and especially in the median and dorsal area of the part known as the concha of the vestibule. One obtains the impression that the entire process in this and other parts of the nasal cavities (olfactory portion, respiratory portion) (Figs. 8, 9, and 10) begins in the mucous membrane epithelium and that the epithelium of the glands becomes involved a little later.

In addition to numerous keratohyalin corpuscles in the cells of the superficial layers of the newly formed squamous stratified epithelium and the appearance of eleidin (Pasini's method), the following lesions in the nuclei of the keratinizing cells have been encountered in the nasal cavities:—As a rule it has been observed, even in the deeper layers, that marked alterations of the nuclei are coexistent with the formation and appearance of keratohyalin granules in the cytoplasm. There is especially a marked fragmentation and extrusion of nuclear material (nucleoli) and furthermore a relative increase in the volume of the cytoplasm as compared with nucleus (Fig. 11). According to the investigations of Ludford (8), the same lesions may take place in the skin under normal conditions, while in a hypertrophied epidermis, under pathological conditions, these nucleolar changes are still more marked. It seems very probable, therefore, that the nuclear substances and particularly the nucleoli are connected in some way with the process of keratinization and the formation of keratohyalin granules. In addition the nucleoli after extrusion from the nuclei not infrequently change their staining properties. When stained by Hammerschmidt's method they resemble Guarnieri's inclusion bodies. Moreover, the nuclei of these cells from which the nucleoli have been extruded have the characteristic shape of nuclei in cells containing Guarnieri's bodies. It is worthy of note that in the pathological keratinization associated with chicken-pox Eberbeck (9) found the same type of lesion.

The sinuses communicating with the nasal cavity show essentially the same lesions as in the epithelium of the nose and trachea. These lesions are modified depending upon the anatomical structure of the cavity itself. In the paranasal sinuses the mucous membrane is thinner and contains only a few glands on the median wall, contrary to the opinon of Bittner (10). Keratinization occurs in the nasolacrimal duct and in the excretory duct of the lateral nasal gland, glandula lateralis nasi. The lateral nasal gland itself is only slightly involved, the main lesion being in the ducts and collecting space while the acini themselves appear normal.

Bacterial invasion of the degenerating epithelium occurs in the nose just as it does in the trachea (Fig. 12). It is, however, more marked and the nasal cavity and fossa may become filled with degenerated cells and purulent exudate. The tunica propria becomes involved and in addition to the infiltrating cells one may find a slight edema and a swelling of the endothelial cells of the blood vessels.

Relation of A-Avitaminosis to Some Infectious Diseases of the Upper Respiratory Tract

The great variability of the clinical symptoms and pathological lesions in A-avitaminosis of the respiratory tract may make it difficult to differentiate this condition from coryza contagiosa, virus-diphtheria, and infectious tracheo-bronchitis. In the presence of a simple nasal discharge and a discharge from the cleft palate, with involvement of the sinuses, and in the absence of typical pustule-like lesions in the mouth and esophagus, there is a close resemblance to coryza contagiosa as well as to a certain stage of virus-diphtheria (Riedmüller (11)). Recent observations have shown that in such cases, from the clinical, gross anatomical, and even etiological aspect, a differentiation is quite impossible. Furthermore in later stages, after the sinuses and the eyes have become involved and when lesions are present in the cavity of the mouth (12), the A-avitaminosis frequently is almost indistinguishable from the commonly occurring roup and virus-diphtheria. The white color of the caseous masses associated with A-avitaminosis is usually considered to be valuable for the differentiation from the more yellowish products in roup and virus-diphtheria; yet these differences, according to our experience, are by no means distinct enough. Finally, in the absence of typical lesions in other organs, difficulties may arise in distinguishing the disease from infectious tracheo-bronchitis which in certain stages shows a very similar picture in the larynx, trachea, and bronchi.

It will be the object of further investigations to discover possible relations between A-avitaminosis and the development of these infectious diseases of the upper respiratory tract. In this problem, as well as for the purposes of differential diagnosis, the most important matter is a positive recognition of a present lack of vitamin A. The histological lesions which occur in the respiratory tract and throughout the body even in early conditions, taken in connection with the more recent investigations of Wolbach and Howe (5), Goldblatt and Benischek (3), and Tyson and Smith (4), who were able to produce similar lesions in rats and guinea pigs by feeding a diet lacking vitamin A, seem definitely to prove the specificity of these lesions. It may be added that in cases of so-called "coryza contagiosa" without a lack of vitamin A quite different lesions have been found.

Only by means of the histological examination is it possible to differentiate the A-avitaminosis from the above mentioned infectious diseases of the respiratory tract.

SUMMARY AND CONCLUSIONS

1. The principal tissue changes in the respiratory tract of chickens caused by a vitamin A deficiency in the food are, first, an atrophy and degeneration of the lining mucous membrane epithelium as well as of the epithelium of the mucous membrane glands. This process is followed or accompanied by a replacement or substitution of the degenerating original epithelium of these parts by a squamous stratified keratinizing epithelium. This newly formed epithelium develops from the primitive columnar epithelium and divides and grows very rapidly. The process appears to be one of substitution rather than a metaplasia, and resembles the normal keratinization of the skin or even more closely the incomplete keratinization of the mucous membranes (*e.g.*, the esophagus or certain parts of the tongue of chickens). In this connection findings have been described which not only afford an interesting insight into the complicated mechanism of keratinization, but also show probable relations between keratinization and the development of Guarnieri's inclusion bodies. Balloon and reticular degeneration of the upper layers of the new stratified epithelium has been frequently observed. All parts of the respiratory tract are about equally involved in the process; and the olfactory region as well, so that the sense of smell may be lost. The lesions, which first take place on the surface epithelium and then in the glands, show only minor differences.

2. The protective mechanism inherent in the mucous membranes of

the entire respiratory tract is seriously damaged or even entirely destroyed by the degeneration of the ciliated cells at the surface and the lack of secretion with bactericidal properties. Secondary infections are frequently found, and nasal discharge and various kinds of inflammatory processes are common, including purulent ones, especially in the upper respiratory tract, communicating sinuses, eyes and trachea. The development of the characteristic histological process is not dependent upon the presence of these infections, since it also takes place in the absence of infection.

3. The specific histological lesions make it possible to differentiate between A-avitaminosis and some infectious diseases of the respiratory tract.

These studies we hope will serve as a basis for further investigations on the relationship between A-avitaminosis and infection in general.

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EXPLANATION OF PLATES

PLATE 21

FIG. 1. Sagittal section through the head of Chicken 7 with A-avitaminosis. Masses of purulent exudate in the nasal passages. (Dead after 82 days on experimental diet.)

FIG. 2. Cross section through the head of Chicken 13 with A-avitaminosis (spontaneous case). White caseous masses between left concha inferior and nasal septum and in the corresponding sinuses.

FIG. 3. Trachea of Chicken 6 with A-avitaminosis. Thin membranes, partly in the form of a tube, consisting of desquamated epithelium. (Dead after 87 days on experimental diet.)

FIG. 4. Trachea, cross section (84.5 ×). Chicken 1. Beginning stage. Degeneration of the surface epithelium and separation from its underlying support. On the left side formation of a new stratified epithelium in a small focus. (Killed after 30 days on experimental diet.)

FIG. 5. Trachea, cross section (50 ×). Chicken 8. Complete replacement of the epithelium of the respiratory mucosa and its glands by a stratified keratinizing epithelium. Infiltration of the submucosa. (Dead after 80 days on experimental diet.)

PLATE 22

FIG. 6. Same as Fig. 5 (80 ×).

FIG. 7. Cross section through trachea (990 ×). Chicken 1. Newly formed stratified epithelium. At the surface several cells showing balloon degeneration. Foci of bacteria between the degenerating cells. (Dead after 30 days on experimental diet.)

FIG. 8. Nasal septum (50 ×). Chicken 6. Complete replacement of the surface epithelium by stratified keratinized epithelium. Desquamation of the superficial layers. The glands show an earlier stage of the process, atrophy of the original epithelium. Slight infiltration of the submucosa. (Dead after 87 days on experimental diet.)

FIG. 9. Olfactory region (85 ×). Chicken 6. Advanced keratinization and desquamation of the replacing epithelium at the surface of the mucous membrane. The epithelium of Bowman's glands shows only the typical atrophy. Masses of exudate and desquamated epithelium. (Dead after 87 days on experimental diet.)

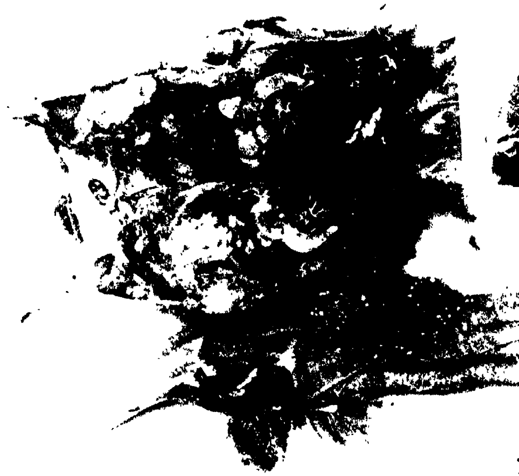
PLATE 23

FIG. 10. Respiratory and olfactory mucous membrane of the nasal fossa (55 ×). Chicken 5. Complete replacement by stratified keratinizing epithelium. The glands show an earlier stage of the process. (Dead after 85 days on experimental diet.)

FIG. 11. Nasal passages. Chicken 6. Cells of the superficial layers of the newly formed keratinizing epithelium, showing the extrusion of nuclear material into the cytoplasm (a), granules of keratohyalin (b), masses of eleidin (c), and intercellular bridges (d).

FIG. 12. Gland of the nasal mucous membrane (1140 ×). Chicken 5. Showing numerous bacteria in an early stage of the process. (Dead after 85 days on experimental diet.)

PLATE 21



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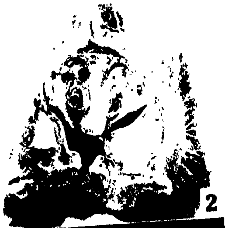

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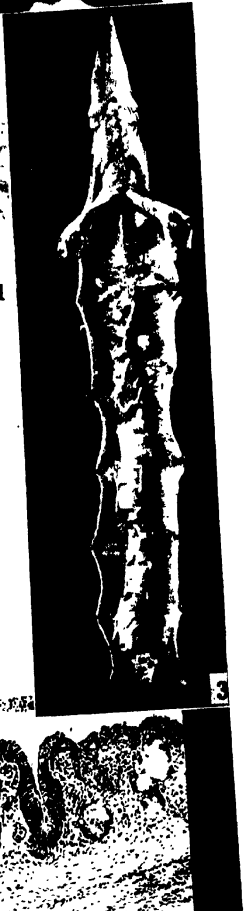

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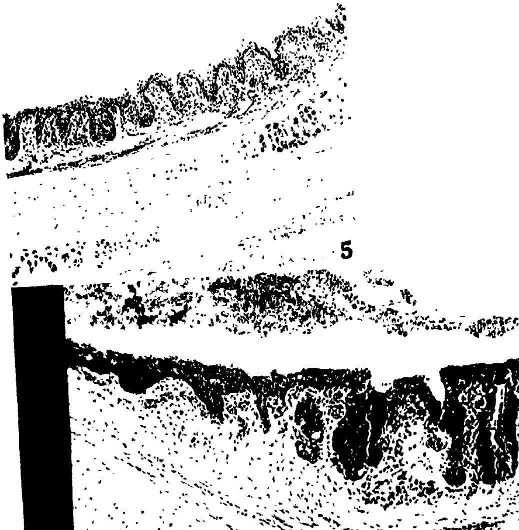

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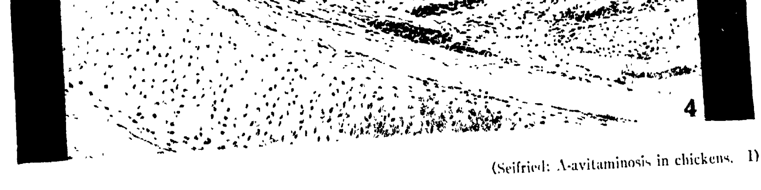

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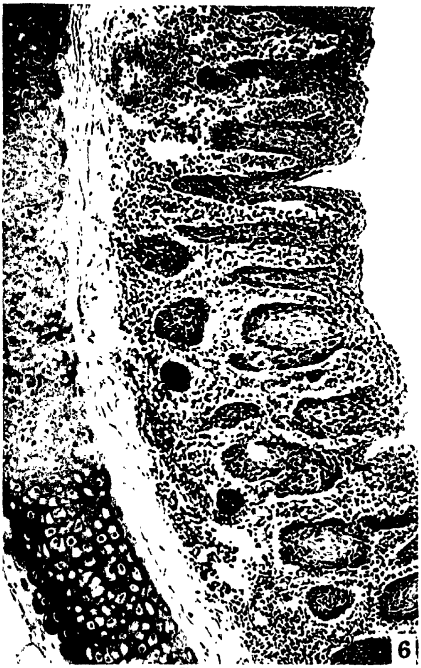

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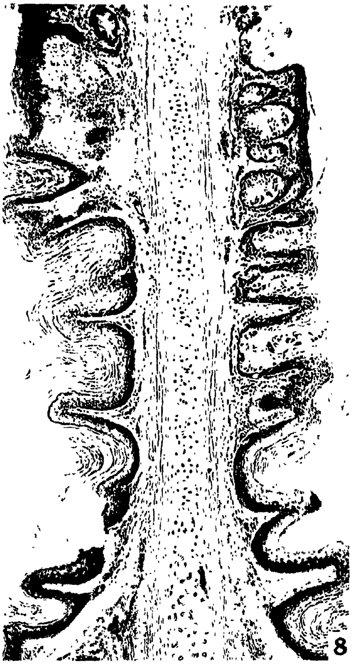

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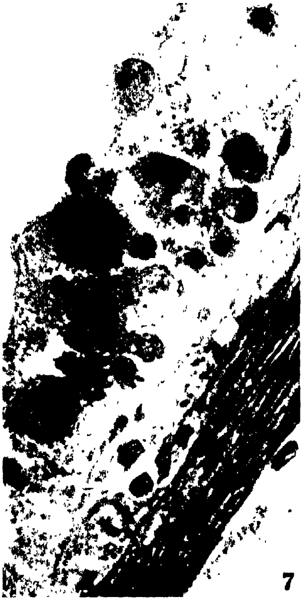

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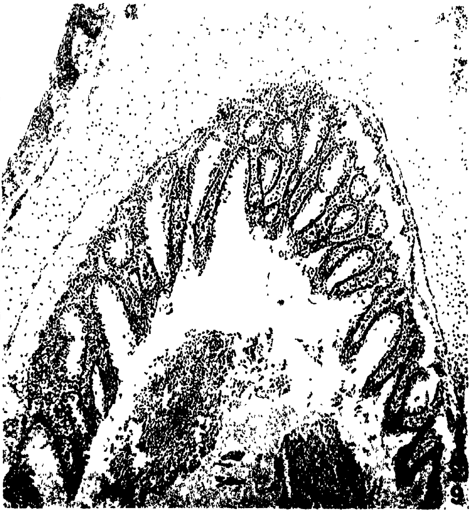

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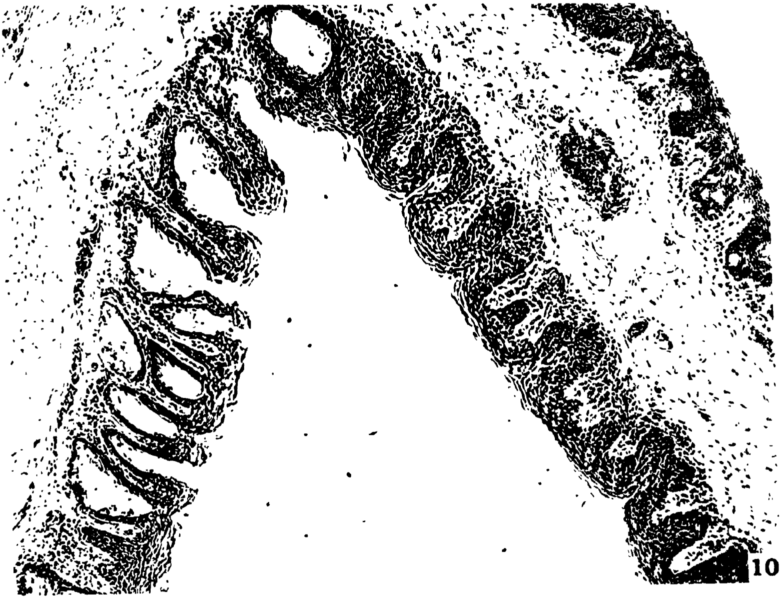

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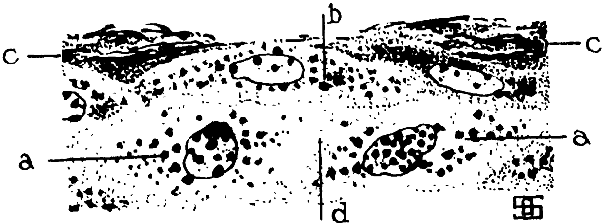

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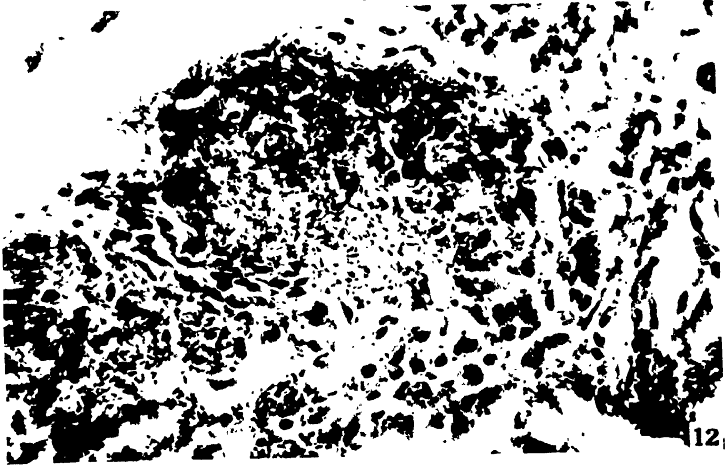

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STUDIES ON A-AVITAMINOSIS IN CHICKENS

II. LESIONS OF THE UPPER ALIMENTARY TRACT AND THEIR RELATION TO SOME INFECTIOUS DISEASES

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PLATES 24 AND 25

(Received for publication, June 19, 1930)

In the previous paper[1] of this series the lesions in the respiratory tract caused by a lack of vitamin A in the food of chickens was considered, together with a discussion of the clinical findings and their relation to certain infectious diseases. Using the same material and methods, it is the purpose now to deal in the same way with the lesions of the mouth, palate, esophagus, crop, and their associated glands.

Gross Pathology

Because of the close connection between the nasal cavity and the mouth, the seromucous discharge in the former overflows regularly through the cleft palate and produces a clinical and anatomical picture which cannot be differentiated from that found in coryza contagiosa. As secondary invaders appear this exudate becomes transformed into white or slightly yellowish caseous masses which may at times completely plug the cleft palate. In addition, in the region of these nasal plugs and also on the roof of the mouth but seldom in the esophagus, fine thin membranes appear which together with the nasal plug produce a picture which may be easily confused with that of fowl-pox. In A-avitaminosis, however, these membranes may not always be present and when they are they usually are limited to the cleft palate and its adjacent epithelium. They are easily removed leaving no bleeding ulceration, thus differing from fowl-pox.

Early in A-avitaminosis there appear small white or yellowish pustule-like lesions, varying from 0.5 to 2 mm. in diameter, in the region of the excretory ducts of the glands of the mouth (Text-figs. 1 and 2). When discrete they are round, but by confluence they may become longish. They are usually raised above the surface of the mucous membrane and often show a depression in the center (Fig. 1). Later small ulcers may appear at the site of these lesions and these ulcers may be surrounded by inflammatory products. These pustule-like lesions, with or without the inflammatory membranes, very closely resemble lesions of fowl-pox, and it is with great difficulty, if at all, that a differential diagnosis can be made by the macroscopical picture.

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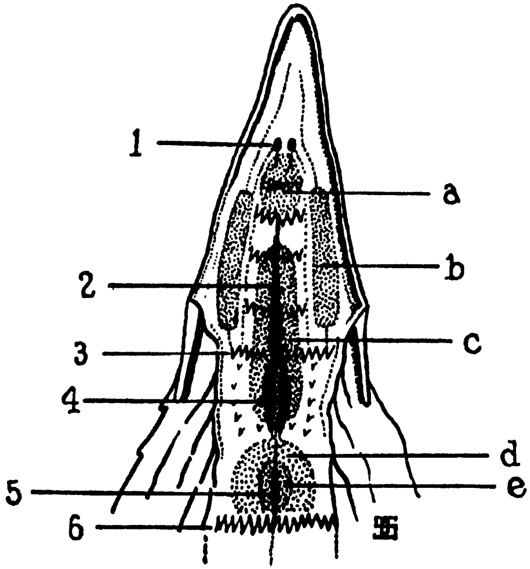
[1] Seifried, O., *J. Exp. Med.*, 1930, **52**, 519.

The mucosa of the esophagus and crop also shows pustule-like patches which may be even more prominent than those seen in the mouth, probably due to the peculiar structure of the glands in these regions (Fig. 1). They may be either localized in the upper part of the esophagus and surrounding the pharynx, or they may cover the mucous membrane of the esophagus throughout its entire length and in cases extend into the posterior part of the crop where the folds of the esophagus are continued. In the main portion of the proventriculus and throughout the intestines no gross lesions have been found. The nodules in the esophagus do not ulcerate as frequently as those in the mouth cavity, but they show a depressed minute opening in the center which corresponds to the excretory duct of the gland. In a few instances they are so numerous in the esophagus that when infected a type of membrane may be formed.

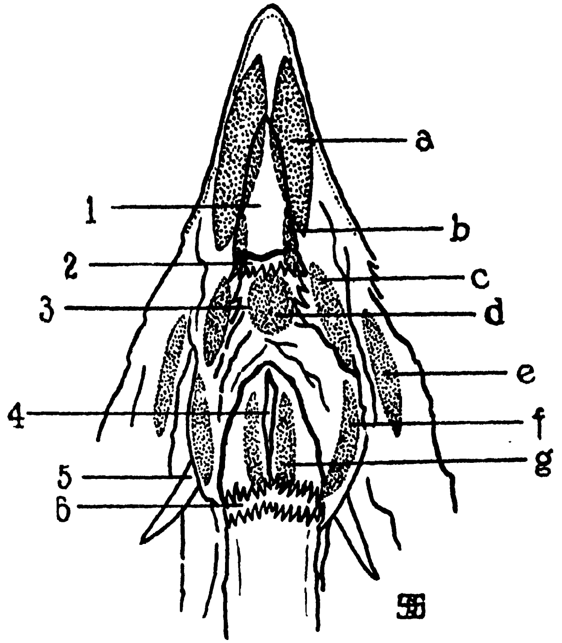


TEXT-FIG. 1. Roof of mouth. 1: excretory ducts of maxillary gland; 2: cleft palate, narrow part; 3: papillae of palate; 4: cleft palate, wide part; 5: infundibulum; 6: papillae of throat. a: glandula maxillaris; b: glandula palatina lateralis; c: glandula palatina medialis; d: glandula spenopterygoida; e: glandula tubaria.

Histology

As noted above the lesions in the alimentary tract occur mainly in the glands. They have been studied in sections made of the roof of the mouth after decalcification.

Glands from the floor of the mouth, and in some instances from the roof, have been dissected out and sectioned in their shortest and longest diameters, while the glands of the tongue, pharynx and esophagus have been sectioned *in situ*. The lesions in the glands are essentially the same as those found in the respiratory tract and described in the previous paper. There are, however, some peculiarities which probably depend upon the special structure of the glands. As a rule the early lesions in these glands are not found in the acini but in the collecting spaces and ducts. Lesions may be well marked in the maxillary and submaxillary glands before they appear in the nasal passages, sinuses, trachea or bronchi. Fig. 2 shows



TEXT-FIG. 2. Floor of mouth. 1: tongue; 2: papillae of tongue; 3: base of tongue; 4: entrance to larynx; 5: bone of tongue; 6: papillae of larynx. a: glandula submaxillaris anterior; b: glandula lingualis anterior; c: glandula submaxillaris oromedialis; d: glandula lingualis posterior; e: glandula submaxillaris caudolateralis; f: glandula submaxillaris intermedia; g: glandula cricoarytenoidea.

an early lesion of the submaxillary gland in which there is a marked proliferation *and degeneration of the original epithelium. At the same time new stratified epithelium appears beneath* or in place of the original epithelium. These early lesions may not be very characteristic but as the process is advanced typical pictures occur, as shown in Fig. 3 of the maxillary gland where in the collecting space is found a focus of squamous stratified keratinizing epithelium. In the acini the high mucus-secreting epithelium has begun to degenerate and atrophy and in a few instances there has been a replacement by stratified keratinizing cells. These changes in the acini have never been as pronounced as in the collecting spaces. Fig. 4, a cross section through the glandula lingualis, shows a well marked stage in the process with newly formed stratified epithelium and early keratinization. This newly formed epithelium may be found in isolated islands or in larger foci beneath or in place of the original epithelium lying directly upon the connective tissue. It seems probable that this newly formed tissue is favored in its growth by the abundant supply of capillaries from the surrounding connective tissue. The collecting spaces become filled with degenerated cells, masses of mucus (Fig. 4), and in some cases inflammatory products. This accumulation is undoubtedly in large part due to the lesions in the excretory ducts of the glands, as it is here that the oldest and most pronounced changes have been found. The epithelium of the mucous membrane of the mouth seems to extend into the duct, which is partially filled with the new stratified epithelium, and causes a more or less complete blocking. As a result the glands become dilated and frequently show evidences of bacterial infection, the latter being especially marked in the duct. Since this is an early lesion the gland may continue to secrete mucus for a time and this accumulates in the collecting space. Later desquamated cells from the newly formed stratified epithelium become numerous and the gland, which originally is a sac with invaginations, becomes smoothed out and distended. Finally this distended sac becomes completely filled with stratified keratinized epithelial cells, as shown in Fig. 5 which gives a picture of the final stage of the process. Such a picture is found more frequently in the glands of the tongue, palate and esophagus than in the compound glands of the cavity of the mouth.

The histological picture shows that early infections of the excretory ducts of the glands are relatively common. These infections are manifested by necrosis of the epithelial lining of the duct and of the surrounding mucous membrane, cellular infiltrations, and the presence of numerous bacteria (Figs. 6 and 7). Oftentimes it appears that the lumen of the duct is blocked, thus resulting in an accumulation of inflammatory products in the gland itself. At times the inflammatory process spreads from the mouth of the duct over the surrounding mucous membrane of the mouth (Fig. 8) or tongue, and more rarely over the mucous membrane of the esophagus.

In the submucosa signs of infection such as perivascular and diffuse infiltrations are frequently seen. In some sections through the tongue such infiltrations have been found even in the deeper layers of the muscularis, and in the ventral portion of the vomer these lesions are particularly pronounced. In the esophagus and crop and sometimes in the proventriculus, the lesions in the glands are the same as those described in the mouth cavity but secondary bacterial infections are not as pronounced.

As pointed out above, the macroscopical lesions in the mouth may closely resemble lesions of fowl-pox. The histological difference between these two is quite striking and there is no difficulty in separating them from sections, but what part the process plays in the development of fowl-pox and other infections by breaking down the resistance of the mucous membranes is a problem that is well worth further consideration.

SUMMARY AND CONCLUSIONS

When fowls are placed on a diet lacking in vitamin A lesions appear in the upper alimentary tract which are confined largely to the mucous glands and their ducts. Histologically it is shown that the original epithelium becomes replaced by a stratified squamous keratinizing epithelium and that secondary infections are relatively common. The ducts of the glands may be blocked leading to distention with secretions and necrotic materials. These lesions macroscopically resemble very closely certain stages of fowl-pox and the two conditions can be separated only by histological examination. It is pointed out that these lesions produced by a lack of vitamin A may enable bacteria and other viruses to enter the body.

EXPLANATION OF PLATES

PLATE 24

FIG. 1. Pustule-like patches at the roof of the mouth, the base of the tongue and the mucous membrane of the esophagus. (Chicken 7, dead after 82 days on experimental diet.)

FIG. 2. Longitudinal section through the glandula submaxillaris of Chicken 11. × 55. Proliferation of the original epithelium lining the collecting space; first stage of process. Hematoxylin-eosin. (Killed after 56 days on experimental diet.)

FIG. 3. Cross section through the maxillary gland of Chicken 1. × 110. Small foci of stratified keratinizing epithelium in the collecting space; degeneration of the epithelium in the acini. Hematoxylin-eosin. (Killed after 30 days on experimental diet.)

FIG. 4. Cross section through the base of the tongue (glandula lingualis) of Chicken 9. × 80. Shows a more advanced stage of the process. The cells, which are to replace the original epithelium, form a continuous vascularized syncytium, lying directly upon the surrounding connective tissue. Beginning keratinization, desquamation and degeneration of the upper layers of the newly formed epithelium. Hematoxylin-eosin. (Dead after 75 days on experimental diet.)

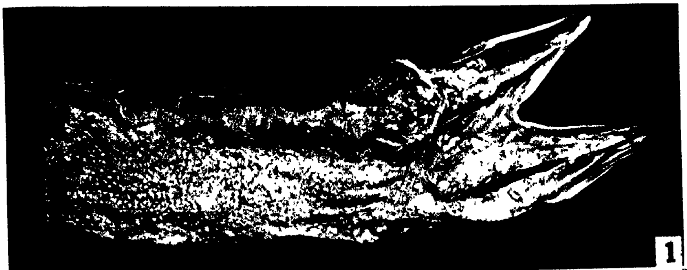
PLATE 25

FIG. 5. Cross section through the base of tongue (glandula lingualis) of Chicken 6. × 50. Final stage of the entire process. The glands are completely filled with stratified, more or less keratinized, homogeneous masses. Extreme dilatation of the glands. van Gieson stain. (Dead after 87 days on experimental diet).

FIG. 6. Excretory duct of the glandula palatina medialis of Chicken 5. × 50. Bacterial infection followed by necrosis of the duct and its surroundings; accumulation of necrotic masses, mucus, inflammatory products and numerous bacteria. Giemsa stain. (Dead after 85 days on experimental diet.)

FIG. 7. Excretory duct of the lateral glandula palatina of Chicken 7. × 875. Invasion with numerous bacteria. Methylene blue stain. (Dead after 82 days on experimental diet.)

FIG. 8. Cross section through roof of mouth (glandula maxillaris) of Chicken 7. × 75. Thick membrane covering the mucous membrane, developing after secondary infection of the excretory ducts. Hematoxylin-eosin. (Dead after 82 days on experimental diet.)



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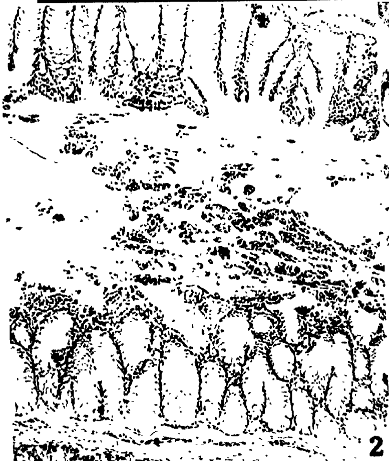

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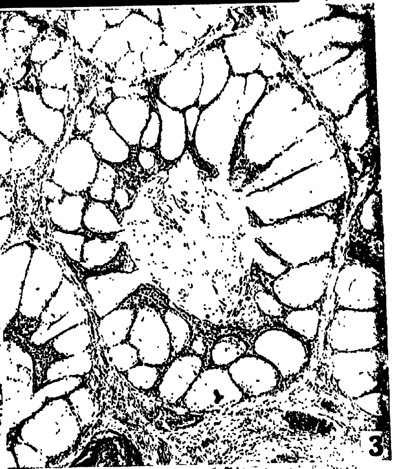

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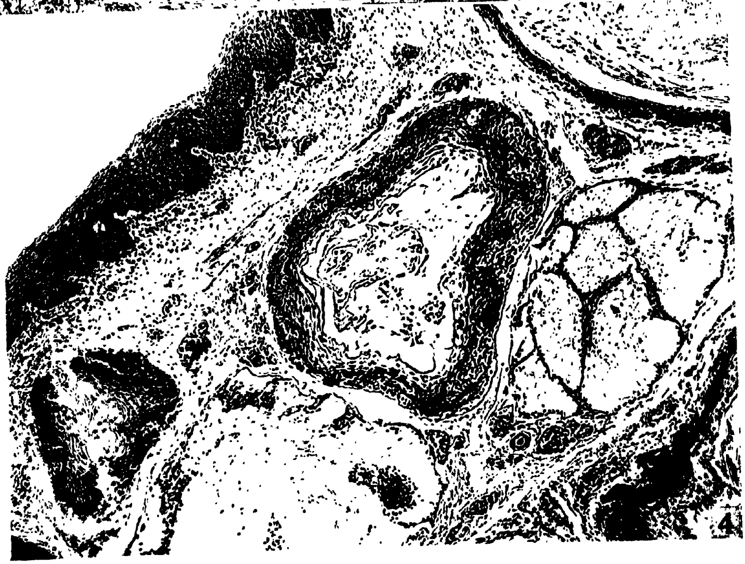

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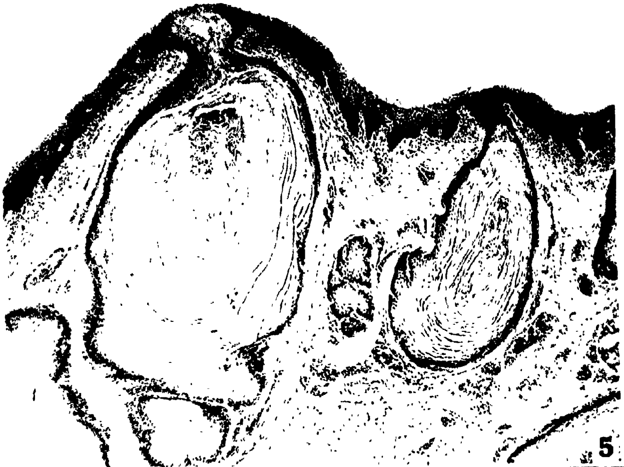

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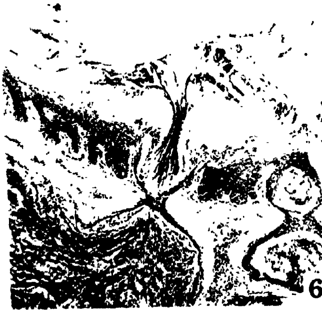

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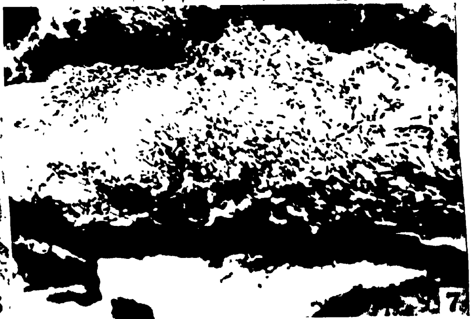

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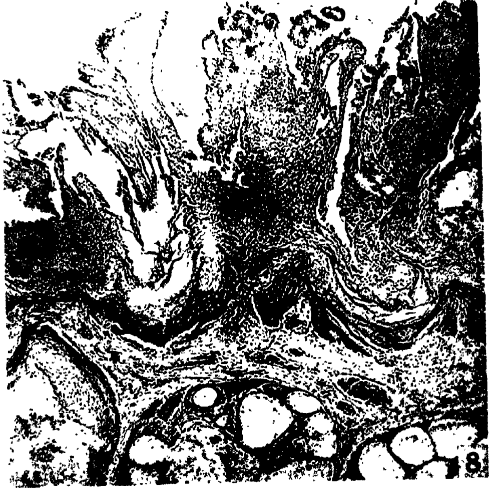

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(Seifried. A-avitaminosis in chickens. II)

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THE ARGININE AND PREARGININE GROUPS IN EDESTIN

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(Accepted for publication, June 22, 1930)

I

INTRODUCTION

Evidence was given in 1928[1] to show that the combination of strong acid or alkali with a protein is produced by forming salts with the "extra groups" of those trivalent amino acids which can be isolated from the protein (namely, aspartic acid, glutamic acid, tyrosine, histidine, lysine, and arginine). The "titration curve" of a protein corresponds approximately to the amounts of these various groups, each with its typical titration index.[2]

Arginine, however, formed a marked exception. It appeared to contribute to the titration curve in much smaller amount than is found on hydrolysis. On the other hand, a group of unknown origin having a titration index of pH 4.6 was present in amounts equal to the deficiency in arginine.

The name *prearginine* was given to this group on the assumption that it is a chemical group which ionizes at pH 4.6 in proteins but yields *arginine* on hydrolysis of the protein.

It has subsequently developed that the index 8.1 assumed for the "extra group" of *arginine* was incorrect (being based on data obtained

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[1] Simms, H. S., *J. Gen. Physiol.*, 1928, **11**, 629; 1928, **12**, 231.

[2] A "dissociation index" is: $pK' = -\log K'$ where K' is the apparent dissociation constant of a weak electrolyte (referred to the hydrogen ion). The dissociation index represents the pH value at which the group is half ionized.

The "titration indices" (pG') determine the titration curve and cannot be correctly called "dissociation indices" although there is little numerical difference between the titration constants (G') and the dissociation constants (K'). The use of these two sets of constants and indices is confusing but unavoidable. See Simms, H. S., *J. Am. Chem. Soc.*, 1926, **48**, 1239.

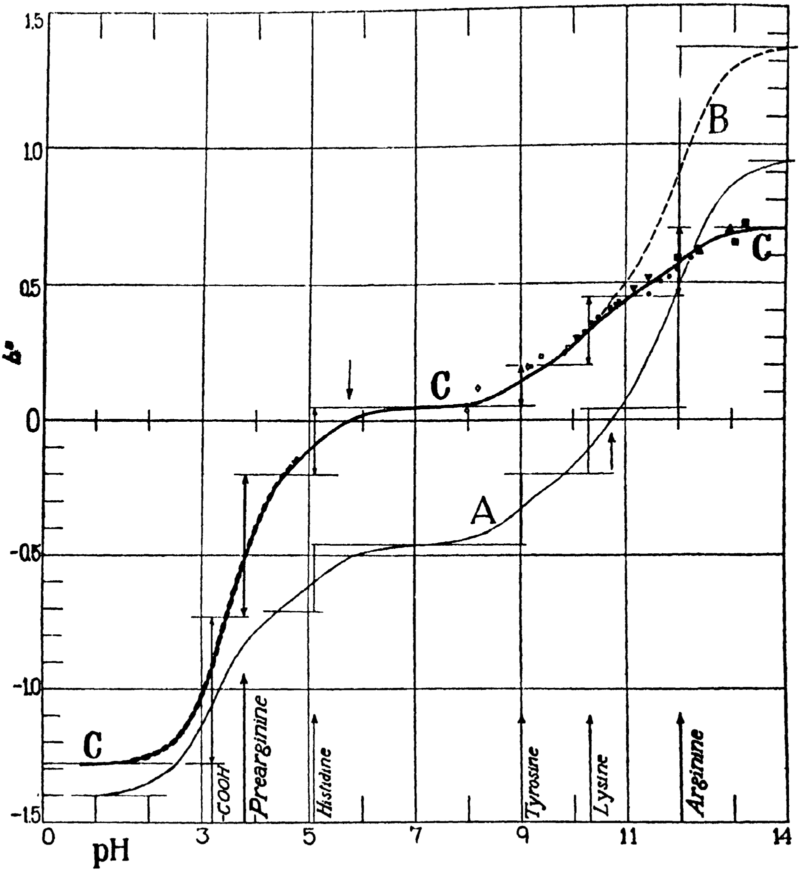

FIG. 1. Titration curve of edestin. The solid symbols represent data from clear solutions; the open symbols, from cloudy solutions. The heavy dash line is drawn to fit the data in acid solution of Hitchcock and of Kodama.

Curve *A* is calculated from the amino acids found on hydrolysis.

Curve *B* (which branches off from *C*) represents the way edestin would ionize if it had the above groups (found on hydrolysis), but in addition had an *extra* group at pH 3.8.

Curve *C* corresponds with the data in Column 5 of Table I.

from impure material). Schmidt[3] has obtained the value pK'_3 = 12.5 which agrees with that of Hunter and Borsook (pK'_3 = 13.85 at 18°C.).

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[3] Schmidt, L. A., Kirk, P. L., and Appleman, W. K., *J. Biol. Chem.*, 1930, **88**, 285.

We have repeated our titration of arginine and corroborate the value of Schmidt.

In order to determine whether this would alter the above theory it was necessary to obtain more accurate data than were available, on a protein in very alkaline solution. Edestin was again chosen due to its high content in arginine.

II

RESULTS

A number of experiments were performed with edestin in solutions up to 14 per cent and at pH values up to 13.5. A method was adopted which as far as possible caused the unavoidable errors to cancel out. The data are given in Fig. 1 (and Table II).

An analysis of these data is given in Table I.

TABLE I

(1)	(2)	(3)	(4)	(5)	(6)
			Equivalents per 1000 gm.		
Type	Groups	Indices in edestin	On hydrolysis	Titration data	Difference
Acidic	Dicarboxylic acids	$pG_1' = 3.2$	0.72	0.55	−0.14
	Tyrosine	$pG_4' = 9.0$	.25	.15	−.10
	Sum		0.97	0.70	
Basic	Prearginine	$pG_2' = 3.8$	0	0.53	+0.53
	Histidine	$pG_3' = 5.1$	0.25	.25	0
	Lysine	$pG_5' = 10.3$	.25	.25	0
	Arginine	$pG_6' = 12.0$	.90	.25	−0.65
	Sum		1.40	1.28	
Sum of prearginine plus arginine			0.90	0.78	

Values in Column 4 are those listed by Mitchell and Hamilton,[4] except for histidine and lysine in which case the values of Van Slyke[5] are taken. The figure for dicarboxylic acids represents aspartic acid (0.76) plus glutamic acid (1.31) minus ammonia (1.35) and represents the carboxyl groups not bound by ammonia.

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[4] Mitchell and Hamilton, The Biochemistry of the Amino Acids, Chem. Cat. Co., 1929, 191.

[5] Van Slyke, D. D., *J. Biol. Chem.*, 1911, 10, 15.

If edestin contained the free groups found on hydrolysis (Table I, Column 4) it would have a titration curve represented by Curve *A* in Fig. 1. This is obviously not the case.

If edestin had approximately the above free groups, and *in addition* a group with an index at pH 3.8, in proper amount, the titration curve would be that found experimentally up to pH 10.5; but above that it would have Curve *B*. This is also not the case.

The experimental data agree well with Curve *C* which corresponds to the values in Column 5 of Table I. *This supports the theory that the basic group in proteins having a titration index at pH 3.8 to 4.6* (depending on the protein) *is due to a group which yields arginine on hydrolysis* and may be properly called *prearginine.*

III

EXPERIMENTAL

The two experiments, recorded in Tables II and III, were performed in water-jacketed hydrogen electrode cells[6] with a saturated KCl liquid junction. A separate solution was made up, from the mother solution, for each determination. The cells were standardized against 0.100 M HCl (pH 1.075).

The four experiments in Tables IV to VII were performed with considerably higher concentrations of edestin (as high as 14 per cent) and it was necessary to use a titrating electrode with agar-KCl junction. In each experiment all the readings were taken on a single solution to which molar NaOH (or HCl) was added with a weighing burette.

Owing to the large correction for hydroxyl ion concentration to be made above pH 13, it was not found satisfactory to standardize the electrodes against HCl. Hence the electrodes were standardized against 0.05 M NaOH before (and sometimes after) each experiment. In Fig. 2 are the values of pH $-\log oh$ for NaOH[7] as determined with the water-jacketed (liquid junction) cells. By using this chart as a standard of pH $-\log oh$ in correcting the edestin data, any errors due to the standard cell, the sodium hydroxide solution or the condition of the electrodes, would be eliminated in the calculation of $\frac{h - oh}{c}$.

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[6] Simms, H. S., *J. Am. Chem. Soc.*, 1923, 45, 2503.

[7] *oh* represents hydroxyl ion *concentration* in moles per liter. The value of pH $-\log oh$ is equal to $\mathrm{pK_W} + \log f_{\mathrm{OH}}$ where f_{OH} is the activity coefficient of the hydroxyl ion (namely, $f_{\mathrm{OH}} \times oh = \mathrm{OH}$ = hydroxyl ion *activity*).

This method does assume, however, that solutions of the same ionic strength have the same liquid function potential and dielectric constant. The ionic strength of the protein solutions will be discussed below.

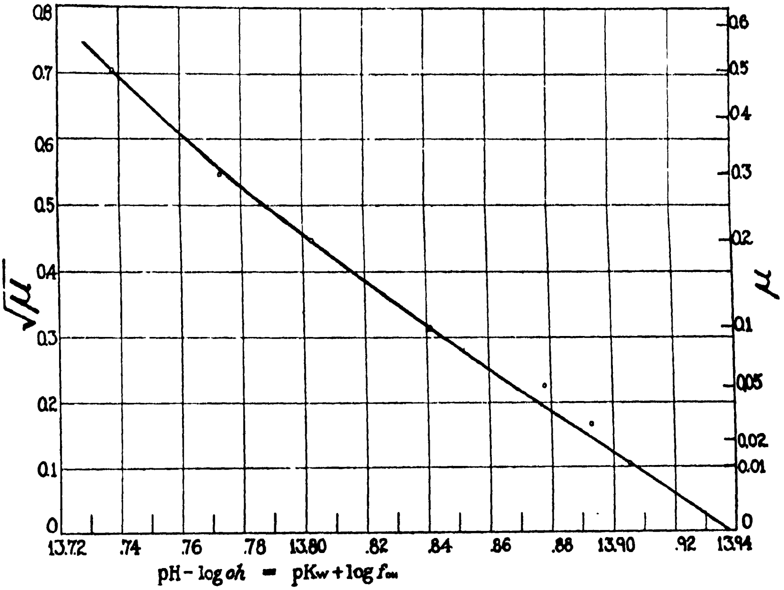

FIG. 2. *Data of the titration of NaOH.* Assuming complete dissociation, the *concentration* of hydroxyl ion (*oh*) is taken equal to the molarity of NaOH.[7]

IV

Calculations

A. Equivalents of Combined Base

The data in Tables II to VII were calculated as follows:

(1) The volumes of *strong base* (V_B) or *strong acid* (V_A) were calculated from the weight of the standard molar solutions, using the density at 25°C.

(2) The volume of *solution* (V) was taken as the sum of the volumes of water plus strong base (plus strong acid when present) and represents *protein-free* volume.

(3) The number of *millimoles* of strong base *minus* ac [illegible] l is:

$$1000\ (B - A) = 1000\ (V_B\ M_B - V_A \quad (1)$$

where M_B and M_A are the moles per liter in the [illegible] ase and acid solutions.

(4) The number of millimoles *per liter* is:

$$b - a = \frac{1000\ (B - A)}{V} \quad (2)$$

(5) The *concentration* in moles per liter of a substance [illegible] molecular weight M is:

$$c = \frac{1000\ W}{M\ V} \quad (3)$$

where W is the weight. But in dealing with a protein or any other substance of uncertain molecular weight it is more convenient to substitute 1000 for the molecular weight (giving us a final value of equivalents per 1000 gm., or milli-equivalents per gram):

$$c' = \frac{W}{V} \text{ gm. per ml.} = \frac{c\ M}{1000} \quad (3a)$$

(6) The number of *equivalents* of strong base minus acid is:

$$\frac{b - a}{c} = \frac{M\ (B - A)}{W} \text{ equivalents added per mole} \quad (4)$$

Or, for a protein:

$$\frac{b - a}{c'} = \frac{1000\ (B - A)}{W} \text{ equivalents added per 1000 gm.} \quad (4a)$$

(7) The ionic strength (μ) was calculated as explained below and used with the curve in Fig. 2 to obtain the value of pH $-$ log oh. The experimental pH value was subtracted from this and the value of $\frac{h - oh}{c'}$ was calculated (where h may be neglected in alkaline solutions).

(8) The "corrected equivalents of base," or the "combined base," is:

$$b' = \frac{b - a}{} + \frac{h - oh}{} \text{ equivalents combined per mole} \quad (5)$$

Or, for a protein:

$$b'' = \frac{b - a}{c'} + \frac{h - oh}{c'} \text{ equivalents combined per 1000 gm.} \qquad (5a)$$

$$b'' = b' \text{ when } M = 1000$$

B. Calculation of Ionic Strength

If we take the experimental data for the effect of ionic strength on protein ionization and assume a point charge, we can calculate an "apparent valence" (ν_A) which is not much greater than unity.[8] This is because the charges on the ionized groups in a protein molecule are so distant from each other that they act almost like separate monovalent ions (*i.e.*, *separate* point charges). The value $\nu_A = 1.8$ was found in acid solutions of gelatin, and $\nu_A = 2.4$ in alkaline solutions.

By using the same assumptions (of point charge) we can use this apparent valence to calculate the approximate contribution of the protein to the ionic strength of its solution. In alkaline solution the contribution of the protein and its bound cation will be:

$$\mu_0 = \frac{\nu_A^2 + \nu_A}{2\,\nu_A}\, b''\, c' = \frac{\nu_A + 1}{2}\, b''\, c' = 1.7\, b''\, c'$$

while the contribution of the unbound NaOH will be given by the hydroxyl ion *concentration*, hence:

$$\begin{aligned} \mu &= 1.7\, b''\, c' + oh + a \\ &= 1.7\,(b - a - oh) + oh + a \\ &= 1.7\, b - 0.7\, oh - 0.7\, a \end{aligned}$$

The data of edestin were calculated in this manner and confirmed the findings with gelatin that the contribution of protein to the ionic strength is even less than that given by this approximation. The results in Tables II to VII and Fig. 1 were calculated by assuming $\nu_A = 1$ (*i.e.*, infinitely distant point charges), hence:

$$\mu = b''\, c' + oh + a = b$$

(where the amount of strong base b, being greater than the strong acid a, includes all free inorganic salt which may be present).

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[8] Simms, H. S., *J. Gen. Physiol.*, 1928, **11**, 613.

TABLE II

Titration of edestin in jacketed electrodes with saturated KCl liquid junction at 25°C. (pH standard: 0.100 M HCl has pH 1.075).

Each solution contained 0.025 gm. edestin, plus 0.015 millimole of NaOH, plus the indicated volume of 0.0200 M HCl and was made up to 10.0 ml. $c' = 0.0025$ and $\mu = 0.0015$. All the solutions were cloudy.

V_A	pH	$\frac{b-a}{c'}$	b''
0.40	9.848	0.280	0.243
.50	9.135	.200	.193
.60	8.181	.120	.119
.70	7.993	.040	.039

TABLE III

Titration of edestin in jacketed electrodes with saturated KCl liquid junction at 25°C. (pH standard: 0.100 M HCl has pH = 1.075).

Each solution contained 0.0625 gm. edestin plus 0.0375 millimole of NaOH, plus the indicated volume of 0.0200 M HCl and was made up to 10.0 ml. $c' = 0.00625$ and $\mu = 0.00375$ in each solution.

V_A	pH	$\frac{b-a}{c'}$	b''
0	10.886	0.600	0.431
0.25	10.710	.520	.408
.50	10.482	.440	.374
.75	10.208	.360	.325
.87	10.078	.320	.294
1.00	9.898	.280	.263*
1.12	9.396	.240	.235*
1.25	9.195	.200	.196*

[*] Cloudy.

TABLE IV

Titration of edestin in a titrating electrode with agar-KCl bridge at 25°C. (pH standard: 0.0494 M NaOH has pH = 12.562).

4.996 gm. (W) of edestin in an initial non-protein volume of 35.00 ml. containing 2.420 millimole NaOH. The amount of 0.991 M NaOH is indicated by V_B (determined by weight; d = 1.040 at 25°C.).

V_B	$c' = \frac{W}{V}$	μ	pH	$\frac{b-a}{c'}$	b''
2.442	0.1428	0.069	11.438	0.484	0.457
2.774	.1414	.078	11.672	.550	.503
2.960	.1407	.083	11.807	.587	.523
3.246	.1396	.090	11.979	.644	.547
3.859	.1372	.105	12.216	.765	.591
4.457	.1350	.119	12.372	.884	.627

TABLE V

Titration of edestin in a titrating electrode with agar-KCl bridge at 25°C. (pH standard: 0.0494 M NaOH has pH = 12.562).

2.500 gm. (W) of edestin in an initial non-protein volume of 17.72 ml. containing 1.708 millimole NaOH. The volume of 0.991 M NaOH is indicated by V_B (determined by weight; d = 1.040 at 25°C.).

V_B	$c' = \frac{W}{V}$	μ	pH	$\frac{b-a}{c'}$	b''
1.724	0.1411	0.0964	11.966	0.683	0.569
5.523	.1161	.254	13.040	2.19	0.65
8.619	.1015	.346	13.217	3.41	.72

TABLE VI

Titration of edestin in a titrating electrode with agar-KCl bridge at 25°C. (pH standard: 0.0494 M NaOH has pH = 12.562).

2.500 gm. (W) of edestin in an initial non-protein volume of 18.14 ml. containing 2.168 millimole NaOH. The volume of 0.991 M NaOH is indicated by V_B (determined by weight; d = 1.040 at 25°C.).

V_B	$c' = \frac{W}{V}$	μ	pH	$\frac{b-a}{c'}$	b''
2.188	0.1371	0.109	12.376	0.867	0.615
4.583	.1218	.221	12.936	1.817	.692

TABLE VII

Titration of edestin in a titrating electrode with agar-KCl bridge at 25°C. (pH standard: 0.0492 M NaOH has pH = 12.560).

0.600 gm. (W) of edestin in an initial non-protein volume of 30.45 ml. containing 0.413 millimole NaOH. To this was added 0.991 M HCl in volumes indicated by V_A (determined by weight; d = 1.016 at 25°C.).

V_A	$c' = \frac{W}{V}$	μ	pH	$\frac{b-a}{c'}$	b''
0	0.01971	0.0136	11.423	0.688	0.518
0.072	.01967	.0159	11.144	.570	.481
.139	.01962	.0180	10.841	.458	.414
.196	.01957	.0198	10.383	.365	.349
.230	.01956	.0209	10.083	.308	.300*

* The last solution was cloudy. The other solutions were clear.

V

SUMMARY

The author corroborates the data of Schmidt showing that the dissociation index of the third group of arginine is $pK_3' = 12.5$.

New titration data of edestin have been obtained in very alkaline solutions and show that there is a corresponding group with a titration index of $pG' = 12.0$, but present in much less quantity than can account for the arginine found on hydrolysis. The data support the theory that the combination of strong base or strong acid with proteins is produced by the formation of salts with the "extra groups" of those trivalent amino acids which can be isolated from the protein, with the exception of arginine. Arginine contributes to the titration curve in much smaller amount than is found on hydrolysis. This deficiency in the arginine group may be accounted for by the basic group in proteins having a titration index of $pG' = 3.8$ to 4.6 (depending on the protein), which apparently yields arginine on hydrolysis, and may properly be called *prearginine.*

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